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Belinda Perkins

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PATENT & TRADEMARK OFFICE

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Nichols *et al.*

Group Art Unit: 1647

Serial No.: 10/726,216

Examiner: Allen, Marianne P.

Filed: December 2, 2003

Docket No.: 421/29/2/2

Confirmation No.: 4262

For: PURIFIED AND ISOLATED PLATELET CALCIUM CHANNEL NUCLEIC ACIDS AND POLYPEPTIDES AND THERAPEUTIC AND SCREENING METHODS USING SAME

* *

DECLARATION OF TIMOTHY C. NICHOLS, M.D.
PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

1. My name is Timothy C. Nichols, M.D., and I am Professor of Medicine and Pathology and the Director of the Francis Owen Blood Research Laboratory at the University of North Carolina at Chapel Hill, upon information and belief the assignee for the subject U.S. Patent Application Serial No. 10/726,216. I am also a co-inventor of the subject U.S. Patent Application Serial No. 10/726,216.

2. A true and accurate copy of my *curriculum vitae*, which evidences my expertise and credentials, is attached herewith and labeled **Exhibit A**.

3. I have had an opportunity to review the subject U.S. Patent Application Serial No. 10/726,216, including particularly claims 1-12 and 45-54 as currently pending.

4. I have also reviewed the following documents: the Final Official Action dated September 26, 2007 (hereinafter "the Final Official Action") on the subject U.S. Patent Application Serial No. 10/726,216 by the U.S. Patent and Trademark Office (hereinafter "the Patent Office"); the Non-Final Official Action dated January 24, 2007 (hereinafter "the Non-Final Official Action") on the subject U.S. Patent Application Serial No. 10/726,216 by the Patent Office; Kunzelmann-Marche *et al.* (2001) 276 J Biol Chem 5134-5139 (hereinafter "Kunzelmann-Marche *et al.*"); and U.S. Patent Application Publication No. 2002/0165353 of Malouf *et al.* (hereinafter "Malouf"), of which I am also a co-inventor.

5. Kunzelmann-Marche *et al.* employ a malignant cell line (human erythroleukemia (HEL) cells) in an effort to document that chemicals that block store-operated Ca^{2+} entry (SOCE) reduce phosphatidyserine (PS) exposure on those cells. *See* the Abstract, lines 10-13.

6. Kunzelmann-Marche *et al.* thus does not study platelets, particularly voltage-dependent calcium channels (VDCCs) that are present on platelets.

7. Kunzelmann-Marche *et al.* states that "little is known about the intracellular signals governing SOCE or the Ca^{2+} channels mediating this particular form of Ca^{2+} entry". *See* page 5134, right column, first full paragraph. Kunzelmann-Marche *et al.* uses agonists and antagonists of SOCE and draws conclusions about calcium signaling in HEL cells.

8. Kunzelmann-Marche *et al.* does not discuss the possibility of a VDCC participating in calcium entry into platelets, and thus there is no disclosure in

Kunzelmann-Marche *et al.* that would support linking a VDCC to PS exposure on HEL cells, much less on platelets.

9. As a consequence, it is believed that Kunzelmann-Marche *et al.* would not lead one working in this field to draw the conclusion that if SOCE exists physiologically in HEL cells, then VDCC must also exist as a protein structure, and that VDCC, even if it were present, must also participate in PS exposure on platelets.

10. Furthermore, when a manuscript discussing the subject matter of the present U.S. patent application was originally submitted for publication to the journal Blood, one of the primary concerns of the reviewers was that a malignant cell line (a cell line of megakaryocytic origin called MEG-01) was first employed for some of the studies.

11. This concern was based at least in part on the fact that malignant and/or transformed cell lines are known to express proteins or phenotypes that are not characteristic of a corresponding non-malignant (*i.e.*, normal, native, or wild type) or non transformed cell of the same cell or tissue type. This was deemed especially important for one reviewer, who cited two papers (copies of which are being submitted herewith as **Exhibits B** and **C**) that showed expression of VDCC in malignant or transformed cells but not in the wild type cell of the same origin. Thus, particularly with respect to the transport of Ca^{2+} by VDCCs, it was believed by the reviewer that malignant cells were not predictive of non-transformed cells.

12. All of the data in the subject U.S. Patent Application Serial No. 10/726,216 were generated with native platelets and native megakaryocytes, including the investigations into PS exposure associated with VDCCα1 activity.

13. Kunzelmann-Marche *et al.* only discloses the use of a malignant cell and not native platelets, and further does not provide any evidence or suggestion that SOCE in malignant cells is mediated by and/or associated with a VDCC biological activity.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

Timothy C. Nichols, M.D.

Timothy C. Nichols, M.D.

January 26, 2008
Date

Attachments: **Exhibits A-C**

CURRICULUM VITAE

PERSONAL INFORMATION

Name: Timothy C. Nichols

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Home Address: 103 Wild Turkey Trail, Chapel Hill, NC, 27516

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Date and Place of Birth: July 9, 1952, Leesburg, Virginia

Marital Status: Married, Evelyn Byrd Quinlivan, M.D.

Children: Elizabeth Byrd Quinlivan Nichols, Aug 20, 1990
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EDUCATION

Brown University, 1970-1972
B.S. (Biology), Stanford University, 1972-1974
M.D., Medical College of Virginia, 1974-1978

PROFESSIONAL EXPERIENCE / EMPLOYMENT HISTORY

Professor of Medicine, North Carolina Memorial Hospital, January 2000-present
Professor of Pathology and Laboratory Medicine, North Carolina Memorial Hospital, January 2000-present
Associate Professor of Medicine, North Carolina Memorial Hospital, July, 1992-1999
Associate Professor of Pathology and Laboratory Medicine, North Carolina Memorial Hospital, July, 1992-1999
Assistant Professor of Medicine, North Carolina Memorial Hospital, 1986-1992
Assistant Professor of Pathology, North Carolina Memorial Hospital, 1991-1992
Instructor in Medicine, Department of Internal Medicine, North Carolina Memorial Hospital, 1985-1986
Clinical Instructor, Department of Internal Medicine, North Carolina Memorial Hospital, 1981-1985
Cardiology Fellowship, North Carolina Memorial Hospital, Department of Internal Medicine, 1982-1985.
This fellowship included 1 year (July 1, 1983, to June 30, 1984) of research in the laboratory of Thomas R. Griggs, M.D., supported by National Research Service Award Training Grant Number 5 T32 HL07470-04
Chief Resident, Department of Internal Medicine, North Carolina Memorial Hospital, 1981-1982
Resident, Department of Internal Medicine, North Carolina Memorial Hospital, 1979-1981
Intern, Department of Internal Medicine, North Carolina Memorial Hospital, 1978-1979

CERTIFICATION / LICENSURE

American Board of Cardiology – Interventional Cardiology, November 7, 2001, #82464
American Board of Cardiology, November 20, 1985, #82464
American Board of Internal Medicine, September 16, 1982, #82464
North Carolina Medical License, 1981, #25383
National Boards, July 2, 1979, #195715

PROFESSIONAL SOCIETIES

Member, American College of Physicians
Member, American College of Cardiology
Member, American Heart Association, Thrombosis and Atherosclerosis Councils
Member, International Society of Thrombosis
Member, American Association of Pathologists

Member, American Society of Hematology

HONORS AND AWARDS

1. Recipient of Young Investigator Award, International Society for Thrombosis and Hemostasis, XIth Congress, Brussels, July 1987.

2. James W. Woods Faculty Award, June, 1988.

3. Kenan Fellowship, 1992-1993

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PUBLISHED ARTICLES IN REFEREED JOURNALS SELECTED FOR EDITORIAL REVIEW

1. Editorial for publication number 13: Aster RH. Freeze-dried blood cells: Therapeutic advance or laboratory curiosity? Proc Natl Acad Sci USA 92:2419-2420, 1995
2. Editorial for publication number 14: Mannucci PM. Platelet von Willebrand factor in inherited and acquired bleeding disorders. Proc Natl Acad Sci USA 92:2428-2432, 1995
3. Editorial for publication numbers 21 and 22: Linden RM, Woo SLC. AAVant-garde gene therapy.

Nature Medicine 5:21-22, 1999

4. Editorial for publication number 37 (Blood Plenary Paper): Katherine Ponder. Gene therapy for hemophilia B in dogs: finally prevention of bleeding, but concerns about inhibitors remain. Blood 99: 2635-2636, 2002

5. Editorial for publication number 45 (Blood Plenary Paper): Peter J. Lenting and Jan J. Sixma. DDAVP and interleukin-11: a boosting combination. Blood 102: 415-416, 2003

6. Editorial for publication number 63: Arruda VR. Toward gene therapy for hemophilia A with novel adenoviral vectors: successes and limitations in canine models. J Thromb Haemost. 2006;4:1215-1217

IN PRESS/ACCEPTED/SUBMITTED

1. Milbauer LC, Enenstein J, Roney M, Solovey A, Bodempudi V, Nichols TC, Hebbel RP. BOEC Migration and Trapping In Vivo: A Window into Gene Therapy. Revision submitted 2007.

2. Nichols TC, Busby WH Jr, Merricks E, Sipos J, Rowland M, Sitko K, Clemmons DR. Protease Resistant IGFBP-4 Inhibits IGF-I Actions and Neointimal Expansion in Porcine Model of Neointimal Hyperplasia. Submitted. 2007

NON-REFEREED JOURNALS

1. Griggs TR, Reddick RL, Lamb MA, Bellinger DA, Nichols TC, Read MS, Brinkhous KM: Studies of coronary thrombosis in swine with von Willebrand's disease. In: Swine in Biomedical Research, Vol. 3. Tumbleson, M.E., (ed.). Plenum Press, New York: 1543-1553, 1985.

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109. Scull CM, Kuruvilla R, Fischer TH, Nichols TC. Gene Transfer to Macrophages with Nanoparticle-Loaded Platelets. Blood. 106; Abstract #3043 November 16, 2005

110. Fischer TH, Wolberg AS, Bode AP, Ramer KJ, Nichols TC. Interaction of Recombinant Factor VIIa with Rehydrated, Lyophilized Platelets. Blood. 106, Abstract #3994, November 16, 2005□

111. Hui DJ, Mingozzi F, Sabatino DE, Dillow A, McCorquodale S, Nichols TC, Arruda VR, High KA. T Cell Responses to Canine Factor IX and AAV Capsid Antigens in Hemophilia B Dogs after Intravascular Gene Delivery to Skeletal Muscle. Molecular Therapy. 2006;12(Abstract supplement):S1086

112. Ehrhardt A, Xu H, Dillow AM, Yant SR, Nichols TC, Kay MA. Transposition from a Gene-Deleted Adenoviral Vector Results in Phenotypic Correction in a Canine Model for Hemophilia B. □□ Molecular Therapy. 2006;12(Abstract supplement):S11.

113. Sabatino D, Lange AM, Mucci M, Sarkar R, Dillow AM, Nichols TC, Arruda V, Kazazian HH. Long Term Dose Dependent Correction of Hemophilia A Dogs Using AAV-8 and AAV-9 FVIII Gene Transfer. Blood 2006 (abstract supplement) :S999

114. Jankowitz RG, Chapman H, Merricks E, Kloos M, Dillow A, Nichols TC, Ragni MV. Recombinant

human IL-11 (rhIL-11), Neumega) Increases VWF Activity in Type 1 von Willebrand Disease. Blood 2006 (abstract supplement):S1003

115. Jankowitz RG, Chapman H, Merricks E, Kloos M, Dillow A, Nichols TC, Ragni MV. Recombinant IL-11 Increases VWF and VWF MRNA in Type 1 VWD. Abstract N° O-T-062, XXI ISTH, July 2007.

116. D. Viuff D, Ezban M, Lind V, Dillow A, Raymer R, Merricks E, Nichols T. Effect of rFVIIIa and NN1731 (rFVIIa analogue) on Thromboelastography (TEG) in Whole blood obtained from Hemophilia A dogs. Abstract N° P-W-113, XXI ISTH, July 2007

PATENTS

1. Purified and isolated platelet calcium channel nucleic acids and polypeptides and therapeutic and screening methods using same. US Provisional Patent Application Serial No. 60/258,169. Inventors - Malouf N, Nichols, TC, and Merricks EM.
2. Freeze-dried red blood cells. (Filed 8/02, PCT App. NO PCT/USO02/27898. Inventors Fischer T, Bode A, Nichols, TC, and Read MS. Assignee – UNC-CH and Ecu, Licensed to Hemocellular Therapeutics, Inc
3. Delivery of therapeutic agents with rehydrated lyophilized platelets. (Filed 6/03, US Provisional Patent Application Ser. No. 60.471,005). Inventors – Nichols TC, Bode A, Read MS, and Fischer T. Summary This patent relates to using lyophilized platelets to deliver therapeutics. Assignee – UNC-CH and Ecu, Licensed to Hemocellular Therapeutics, Inc.

TEACHING RECORD AND RESPONSIBILITIES

a. Attending in the cardiac catheterization laboratory: I attended in the cardiac catheterization laboratory for 17 years teaching medical students, radiology students, cardiovascular technologists, medicine housestaff, and cardiology fellows. This work often included providing consultation with referring attendings inside and outside UNC. In the cardiac catheterization laboratory, I was responsible for teaching cardiology fellows all aspects of cardiac catheterization, hemodynamic physiology, patient selection for surgery, balloon and stent angioplasty, and valvuloplasty.

b. Attending on the in-patient cardiology ward and consult service. This assignment requires the attending to mentor and supervise cardiology fellows, housestaff from Internal Medicine and several other Departments and medical students on the in-patient cardiology service. The attending and the fellow also see in-patient cardiology consults when the consult service is unavailable (each weekend, holidays, nights).

c. Cardiovascular Radiology lectures to the Radiology housestaff: One to three lectures per year to the radiology residents on cardiovascular radiographic anatomy and pathology 1997- 2000.

e. Graduate students on whose thesis committee I currently serve or have served:

Department of Pathology	Date Completed
1. Alyssa A Gulledge	1999
2. Tracey C Dawson	1999
3. Gavin Gordin	2000
4. Jen Wemhoff	2001
Oral Biology	
1. Yong Liu	pending

f. Pathology 213 Mechanisms of Disease, Unit Director for section entitled "Vascular Disease." This is a required course for Pathology graduate students. I have responsible for giving and scheduling lectures and administering and grading the exam for the past 7 years.

g. Mentor for Research Trainees supported on the NIH Training Grant in Experimental Cardiology.
I have been responsible for recruiting and training pre and post doctoral research trainees (list available upon request)

h. Research conferences/seminars at UNC (selected)

Department	Date	Title of Seminar
Department Pathology	November 1997	Genetically determined susceptibility to atherosclerosis in vWD pigs.

i. Department of Medicine Morbidity and Mortality Conference

(i) Morbidity and Mortality conference discussant for "Uremic Pericarditis" March 7, 1995
(ii) CPC Discussant July 30, 1996, in a case of thrombosed prosthetic aortic valve.
(iii) CPC Discussant April 27, 1999, in a case of constrictive pericarditis.

j. AHEC conferences (recent)

Location	Date	Title of Seminar
Moore Regional Hospital	Feb 8, 1996	Coronary Atherectomy
Wake/Dix	April 13, 1999	Atherosclerosis in 1999
Moses Cone	Spring 2000	Atherosclerosis Update
Mountain AHEC	Spring 2002	Lipids and Inflammation in Atherosclerosis
Moses Cone	Spring 2003	Inflammation and Atherosclerosis
Wilmington	Spring 2006	Atherosclerosis and Periodontitis
Wake/Dix	Spring 2007	Atherosclerosis in Insulin Resistant Pig

Summary of Attending Responsibilities That Include Teaching from 2000 through 2006

	2000-2001	2001-2002	2002-2003	2003-2004	2004-2005	2005-2006
1. In-Patient Cardiology Ward and Consults	2 months	3 months	3 months	3 months	3 months	3 months
2. Cardiac Catheterization Laboratory Night Call (25-33.3% of total attending call)	3 months	3 months				
3. Cardiac Catheterization Laboratory Day Time	1 day/week	1 day/week				
4. Cardiology Clinic		1/2 day/wk	1/2 day/wk	1/2 day/wk	1/2 day/month	1/2 day/month
5. ECG reading	1 day/month	1 day/month	1 day/month	1 day/month	1 day/month	1 day/month

SELECTED INVITED SEMINARS AND GRAND ROUNDS PRESENTED AT OTHER INSTITUTIONS, NATIONAL OR INTERNATIONAL MEETINGS 7/1/96 – 2005:

Department/Meeting Name	Location	Date	Role
Department of Reproductive Medicine	NCSU Vet School	11/97	Increased Suceptibility to Atherosclerosis in VWD pigs
70 th Scientific Meetings AHA	Orlando, FL	11/97	Vascular Biology Abstract Session Moderator
National Hemophilia Foundation Gene Therapy Workshop	Scripps Inst La Jolla, CA	11/98	Title: Coagulation assays in the monitoring of gene therapy of hemophilia A and hemophilia B in dogs
National Hemophilia Foundation Gene Therapy Workshop	Washington D.C.	3/00	Title: Absence of circulating F.IX antigen in the Chapel Hill strain of hemophilia B dogs
National Hemophilia Foundation	Salk Institute	4/01	Title: Summary of Gene Therapy in

Gene Therapy Workshop	La Jolla, CA		Hemophilia A and Hemophilia B dogs
American Acad of Periodontology NIDCR	Bethesda, MD	4/01	Title: Role of NF-kappaB in Inflammation, Periodontitis, and Atherogenesis
American Society of Hematology	Orlando, Fl	12/01	Vascular Biology Abstract Session Moderator
Hematology Grand Rounds	Univ. Pittsburg	11/02	Title: Preclinical gene therapy studies and new approaches to treatments for hemophilia and vWD
National Hemophilia Foundation Gene Therapy Workshop	Salk Institute La Jolla, CA	4/03	Title: Sensitivity of Coagulation Assays Used to Monitor Gene Therapy of Hemophilia A and Hemophilia B dogs
2003 Scientific Subcommittees Scientific & Standardization Committee: Animal, Cellular & Molecular Models International Society Thrombosis & Haemostasis	Birmingham, England	7/03	Title: Recurrent bacteremia with *Porphyromonas gingivalis* induces systemic inflammation and atherosclerosis in normocholesterolemic pigs
Hematology Grand Rounds	Univ. of Penn Philadelphia PA	3/04	Title: Molecular basis for new treatments for hemophilia and vWD
National Hemophilia Foundation Gene Therapy Workshop	Univ. of Penn Philadelphia PA	4/05	Title: Molecular Defect and Cytokine-Induced Gene Expression in Canine VWD
2005 Scientific Subcommittees Scientific & Standardization Committee: Animal, Cellular & Molecular Models International Society Thrombosis & Haemostasis	Sydney, Australia	7/05	Title: Recurrent bacteremia with *Porphyromonas gingivalis* induces atherosclerosis in normo and hypercholesterolemic pigs
UNC SOM Frontiers in Medicine Complications of Diabetes Mellitus Clinical Challenges and Research Advances	Chapel Hill	12/05	Atherosclerosis in insulin resistant familial hypercholesterolemic pigs

ORGANIZATION OF NATIONAL OR INTERNATIONAL MEETINGS

Meeting Name	Location	Date	Title of Talk/Session/Symposium
7th Annual Symposium of the Center for Thrombosis and Hemostasis	Chapel Hill	May 2000	Program Organizer for conference entitled: "Etiology and Treatment of Coronary Arterial Thrombosis: Basic Science and New Clinical Approaches"
XVIII Congress International Society Of Thrombosis and Hemostasis	Paris	July 2001	Program Organizer
XIX Congress International Society Of Thrombosis and Hemostasis	Birmingham England	July 2003	Program Organizer

XX Congress International Society Of Thrombosis and Hemostasis	Sydney Australia	July 2005	Program Organizer
UNC SOM Frontiers in Medicine Complications of Diabetes Mellitus Clinical Challenges and Research Advances	Chapel Hill	Dec 2005	Program Organizer

Grants / Other Support

Timothy Nichols, M.D.

ACTIVE

2 R24-HL63098 (Nichols TC, PI) NIH/NHLBI Maintenance of Animal Models of Human Hemophilia and vWD The major goals of this project are to produce and maintain hemophilic and von Willebrand Disease animals for performing independent and collaborative research studies.	07/01/03 - 06/30/08 Annual Direct Costs $499,957	20%
OVERLAP: none.		
R01 HL69364 (Clemmons DR, PI; Nichols TC Co-I) NIH/NHLBI Atherosclerosis in Insulin-Resistant, Hyperlipidemic Pigs. The major goals of this project are to develop and characterize of insulin-resistant atherosclerotic pigs.	09/30/01-08/31/06 Annual Direct Costs $367,325	20%
OVERLAP: none.		
R01 (Ponder K, Washington Univ, PI; Nichols TC, Co-I) NIH/NHLBI Gene Therapy Blood Protein Deficiencies. The major goals of this project are to optimize strategies for gene therapy of hemophilia utilizing liver-directed retroviral vectors.	02/01/02 – 12/31/05 Annual Direct Costs $38,682	5%
OVERLAP: none.		
2P01 HL64190, (High K, Univ. of Penn. PI) NIH/NHLBI Gene Therapy for Hemophilia Core C: Canine Core Role for Nichols TC: Canine Core Director, subcontract at UNC The major goals of this project are to utilize novel AAV vectors to treat hemophilia.	4/1/05-3/31/10 Annual Direct Costs: $113,000	10%
OVERLAP: none.		

U01DE13940 (Offenbacher S, PI; Nichols TC, Co-I) NIH/NIDCR A Periodontal Program to Prevent Cardiovascular Events The major goals of this project are to conduct a pilot clinical trial to determine the role of periodontitis on cardiovascular events.	07/15/01 – 07/14/04 (continued)	0%
OVERLAP: none.		
P20 Sartor B, P.I. Non-invasive Assessment of Inflammation SubProject PI Nichols TC, SubProject Title: Subproject: Identification of Molecular Targets for Noninvasive Detection of Inflammation in Atherosclerosis in FH Pigs	07/1/05 – 06/30/06 $50,000	5%
OVERLAP: none.		
P20 Sartor B, P.I. Non-invasive Assessment of Inflammation SubProject PIs Gallippi C, Nichols TC, Fischer T. SubProject Title: Novel Ultrasound Probes for Molecular Imaging of Vascular Inflammation in Atherogenesis	09/1/05 – 08/31/06 $50,000	5%
OVERLAP: none.		
STTR Clemmons DR, P.I. The Development of Novel Methods for inhibiting atherosclerosis in diabetes. The major goals of this project are to develop and tes novel strategies for inhibiting atherosclerosis in pig models	03/1/06 – 02/28/07 $70,0201 to UNC	5%
OVERLAP: none.		

FUNDED – START DATE PENDING

SBIR (Hackett PB & McIVOR S, U MN, PI; Nichols, TC: Co-I) NIH Sleeping Beauty Mediated Gene Therapy for Hemophilia The major goals of this project are to develop transposon mediated gene therapy for hemophilia.	11/01/05 to 10/31/06 Annual Direct Costs requested for UNC: $120,182 Start date pending.	20%
OVERLAP: none.		

SBIR (Velander W, U Nebraska PI; Nichols TC: Co-I) NIH cGMP Recombinant Human Factor IX in hemophilia B therapy The major goals of this project are to test recombinant F.IX made in pig mammary glands.	04/01/05-03/31/10 Annual Direct Costs requested for UNC: $141,938 Start date pending	15%

OVERLAP: none.

PENDING

R01 Wang, Univ of Penn, P.I.; Nichols, TC, Co-I NIH Pseudotyped AAV Vector for Hemophilia B Gene Therapy The major goals of this project are to utilize novel AAV vectors to treat hemophilia.	7/1/06-6/30/11 Annual Direct Costs requested for UNC: $75,477	10%

OVERLAP: none.

STTR Selzman C. PI, Nichols TC CoI NF-kappaB Inhibition to Prevent Myocardial Reperfusion Injury. The major goals of this project are to test NF-kappaB inhibitors in myocardial reperfusion injury	9/1/06 – 8/30/07 Annual Direct Costs requested for UNC: $106,037	10%

OVERLAP: none.

PROFESSIONAL SERVICE

School of Medicine

1. Medical School Admissions Committee Secondary Reviewer, September 1998 - 1999

UNC-CH

1. Chair, search committee, for assistant professor (EPA 804), December 1990 through April 1991.
2. Search Committee for Director Division of Laboratory Animal Medicine 2002 and 2003
3. P20 Roadmap Executive Committee for P20 RR020764 Noninvasive approaches to assessing inflammation, Balfour Sartor PI, 2004 -
4. Executive Council of the Carolina Cardiovascular Biology CenterScientific Advisory Committee Center for Carolina Cardiovascular Biology Center, WC Patterson, Director, 2005 -

UNC Hospitals

1. Housestaff Council, Chairman, 1981-1982, North Carolina Memorial Hospital
2. Executive Committee, Member, 1981-1982, North Carolina Memorial Hospital
5. Housestaff Faculty Liaison Committee, Member, 1978-1982, North Carolina Memorial Hospital

North Carolina

1. Reviewer: North Carolina American Heart Association Research Committee Grant Reviewer 1988-93.
2. UNC-CH director of American Heart Association Summer High School Fellowship Program 1992 –1999

National and International

1. NIH Study Section: NHLBI Program Project Grant Reviewer 2003
2. Co-chair Animal, Cellular & Molecular Models Subcommittee of the Scientific & Standardization Committee for the International Society Thrombosis & Haemostasis 2001 - current

OTHER ADMINISTRATIVE ACTIVITIES

Associate Director, C.V. Richardson Cardiac Catheterization Laboratory, 1986-1994
Acting Director, C.V. Richardson Catheterization Laboratory, 1987-88.
Associate Director Francis Owen Blood Research Laboratory 1995 - to 1999
Director Francis Owen Blood Research Laboratory (FOBRL) 1999 –
Please see description of FOBRL on next page.

SCIENTIFIC ADIVISORY BOARD MEMBERSHIPS

1. Cell Based Delivery, Providence Rhode Island
2. Wyeth Research, Cambridge MA
3. Colgate Research, New York NY

EXHIBIT B

Journal of Physiology (1989), **412**, *pp.* 135–154
With 11 *text-figures*
Printed in Great Britain

CHARACTERIZATION OF Ca^{2+} AND K^+ CURRENTS IN THE HUMAN JURKAT T CELL LINE: EFFECTS OF PHYTOHAEMAGGLUTININ

BY GILLES DUPUIS*, JOSÉE HÉROUX* AND MARCEL D. PAYET†‡

*From the *Departments of Biochemistry and †Physiology and Biophysics, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada J1H 5N4*

(*Received 1 June 1988*)

SUMMARY

1. Inward and outward currents were recorded in the human Jurkat T cell line using the whole-cell configuration of the patch-clamp technique.

2. The transient outward current was activated at membrane potentials positive to −60 mV. The activation time constant–voltage relationship decreased from 17 ms to 2 ms for membrane potentials ranging from −40 to +40 mV. The inactivation phase could be fitted by a single-exponential function and the inactivation time constant decreased from 250 ms to 150 ms for membrane potentials ranging from −20 to +100 mV.

3. The steady-state inactivation–voltage relationship showed a mid-point potential of $-32 \pm 2{\cdot}6$ mV, and the slope factor was $10{\cdot}8 \pm 1{\cdot}8$ mv ($n = 3$).

4. The calcium ionophore A23187 provoked a decrease in the amplitude of the outward current, suggesting a dependence of this current on the cytosolic concentration of Ca^{2+}.

5. The K^+ outward current was blocked by tetraethylammonium (TEA, Michaelis–Menten constant (K_m), 6 mM) and by the calcium channel blockers Ni^{2+}, Co^{2+}, Mn^{2+} and Cd^{2+}.

6. Forty per cent ($n = 120$) of the patched Jurkat cells displayed an inward current. In a physiological medium containing Ca^{2+} (2·2 mM), the inward current threshold voltage was −60 mV, the maximum current was observed at −40 mV and the zero current voltage was positive to +20 mV. At negative membrane potentials, the time required to reach 50% of the maximum amplitude was 60 ms and grew shorter with increasing depolarization, reaching a value of 5 ms at −5 mV. The inactivation of the inward current was very slow and the time constant varied from 1200 ms at −35 mV to approximately 250 ms for potentials positive to −10 mV.

7. The current availability had a value of one for potentials negative to −50 mV and zero for potentials positive to −15 mV. The mid-point potential was $-31 \pm 3{\cdot}4$ mV and the slope factor was $3{\cdot}3 \pm 0{\cdot}2$ mV ($n = 3$).

8. The inward channels were permeable to Sr^{2+}, but were blocked by classical Ca^{2+} channel inhibitors such as Co^{2+}, Mn^{2+} and Ni^{2+}.

9. *Phaseolus vulgaris* phytohaemagglutinin (PHA), an inducer of interleukin-2 production in Jurkat cells, increased the inward current amplitude by 32 ± 20%

‡ To whom reprint requests should be addressed.

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($n = 4$). This increase was concomitant with a decrease ($45 \pm 12\%$) in the amplitude of the outward current, but only when the current was carried by Ca^{2+}. In the case of cells that did not show a Ca^{2+} inward current, the amplitude of the outward current was increased by $17{\cdot}6 \pm 13\%$ ($n = 5$) at -20 mV.

10. Because PHA stimulation of interleukin-2 production by Jurkat cells depends on an increase in $[Ca^{2+}]_i$, we suggest that this response depends, at least in part, on the stimulation of voltage-dependent Ca^{2+} channels.

INTRODUCTION

The addition of stimulants such as mitogens, monoclonal antibodies or antigens to lymphocytes cultured *in vitro* results in the perturbation of a series of metabolic processes and cell division (MacDonald & Nabholz, 1986; Cambier & Ransom, 1987; Linch, Wallace & O'Flynn, 1987). Significant changes in the rate of cation transport are amongst the earliest detectable events and such changes are related to subsequent lymphocyte proliferation (Kaplan, 1978; Deutsch & Price, 1982; Owens & Kaplan, 1982). Ionic channels must therefore play a determining role in triggering the cascade of events leading to mitosis. The patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) has been used to obtain evidence suggesting that a transient outward K^+ flux may play a key role in cellular response, including lymphocyte activation (DeCoursey, Chandy, Gupta & Cahalan, 1984; Fukushima, Hagiwara & Henkart, 1984; Matteson & Deutsch, 1984; Cahalan, Chandy, DeCoursey & Gupta, 1985). In addition, the use of fluorescent calcium chelators (Tsien, Pozzan & Rink, 1982) has demonstrated that an increase in $[Ca^{2+}]_i$ is an essential part of the triggering signal of lymphocyte stimulation (Weiss, Imboden, Shoback & Stobo, 1984; Weiss, Imboden, Hardy, Manger, Terhorst & Stobo, 1986; Alcover, Ramarli, Richardson, Chang & Reinherz, 1987; Gelfand, Mills, Cheung, Lee & Grinstein, 1987). Michell (1975) originally proposed that Ca^{2+} channels may be responsible for Ca^{2+} entry into the cells, and unitary inward currents, of which the probability of opening is increased by lectins, have recently been recorded (Kuno, Goronzy, Weyand & Gardner, 1986; Kuno & Gardner, 1987). However, macroscopic Ca^{2+} inward currents in human T cells have not been clearly characterized yet, nor has the effect of phytohaemagglutinin (PHA) on this current been studied.

In the present report, we have used the whole-cell configuration of the patch-clamp technique to investigate the electrical properties of leukemic human Jurkat T cells (Schneider, Schwenk & Bornkmann, 1977) and the effect of PHA, a mitogenic lectin that induces IL-2 production in these cells (Gillis & Watson, 1980; Dupuis & Bastin, 1988). Our results show the existence of a voltage-dependent K^+ channel characterized by a rapid activation and a slow inactivation. The properties of this channel are similar to those described for the well-known delayed rectifier K^+ current of nerve and muscle cells. The current amplitude of the K^+ channel is decreased in the presence of the Ca^{2+} ionophore A23187. We also present data that suggest the presence of a voltage-dependent inward current that can be conclusively attributed to Ca^{2+} ions. PHA consistently increased the inward current with a concomitant decrease in outward K^+ current. Our data suggest that the previously reported increase in PHA-dependent $[Ca^{2+}]_i$ in Jurkat cells (Weiss *et al.* 1986) is due, at least

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EXHIBIT B

in part, to voltage-dependent Ca^{2+} channels, whereas outward K^+ current is indirectly mediated through a $[Ca^{2+}]_i$ increase during the early phase of stimulation. In support of this interpretation, PHA increased slightly the outward current in Jurkat cells that did not show a Ca^{2+} current, in agreement with reports of Cahalan *et al.* (1985) and Decoursey *et al.* (1984) in the case of peripheral blood T cells.

METHODS

Materials

Bovine serum albumin (BSA), antibiotics and poly-L-lysine were purchased from Sigma Chemical Co. (St Louis, Mo, USA). Fetal bovine serum and culture media came from Flow Laboratories (Montreal). Gey's balanced salt solution (GBSS) and RPMI 1640 medium were from Gibco (Grand Island Biologicals, Grand Island, NY, USA). PHA from *Phaseolus vulgaris* (red kidney bean) was prepared in our laboratory (Dupuis & Bastin, 1988). It showed one Coomassie Brilliant Blue-positive band by sodium dodecylsulphate–polyacrylamide gel electrophoretic analysis (M_r 33000).

Cell cultures

Jurkat 77 6·8 cells were kindly provided by Dr. K. A. Smith (Dartmouth Medical School, Hanover, NH, USA) and maintained in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (100 μg/ml) and garamycin (40 μg/ml). The medium was supplemented with 10% heat-inactivated (56 °C for 20 min) fetal bovine serum.

Patch-clamp experiments

These were performed with Jurkat 77 6·8 cells resting in a Petri dish that had been coated 24 h earlier with poly-L-lysine (0·1 mg/ml) dissolved in GBSS and washed with GBSS and BSA (1%). The bath solution (GBSS) contained (mM): NaCl, 120; $CaCl_2$, 2·12; KCl, 5; $MgCl_2$, 2·2; $MgSO_4$; 0·6; D-glucose, 5·6; HEPES, 39; at pH 7·4. Experiments were carried out at 20 °C. The patch electrodes were filled with (mM): KCl, 120; NaCl, 20; $CaCl_2$, 1; EGTA, 11; HEPES, 5; at pH 7·3. Where appropriate, KCl was replaced by an equimolar concentration of CsCl to suppress outward current. The pipette resistance (Pyrex glass, Corning No. 7740) ranged from 4 to 8 MΩ, and the seal resistance varied between 10 and 50 GΩ. Once the seal was established, the cell membrane was ruptured by applying negative pressure. Series resistance was corrected empirically until the rigging point was attained. Experiments were performed using an Axopatch 1-B amplifier. Currents were low-pass filtered (1 kHz) and digitalized on-line via a PDP 11/23 computer. After whole-cell configuration was achieved, the system was left for 10–15 min to allow the voltage dependency of various parameters to stabilize. The cells were then stimulated at a low rate (1 stimulus/30 s) to avoid accumulation of inactivation. The leak current was subtracted from the current–voltage curves.

RESULTS

Whole-cell currents

One example of currents, recorded from a single Jurkat T cell maintained in GBSS, is illustrated in Fig. 1*A*. In this case, the holding potential (HP) was −80 mV. As shown in the figure, a membrane potential less negative than −40 mV activated an inward current which was followed by an outward current that increased with further membrane depolarization. The current–voltage relationship, measured at the peak of either the inward or the outward currents, is shown in Fig. 1*B*. The threshold voltage was observed at approximately −40 mV, whereas the maximum current was obtained at a potential of −20 mV. The current is directed outward for potentials positive to +5 mV. These results indicated that outward as well as inward currents could be recorded in the human Jurkat T cell line.

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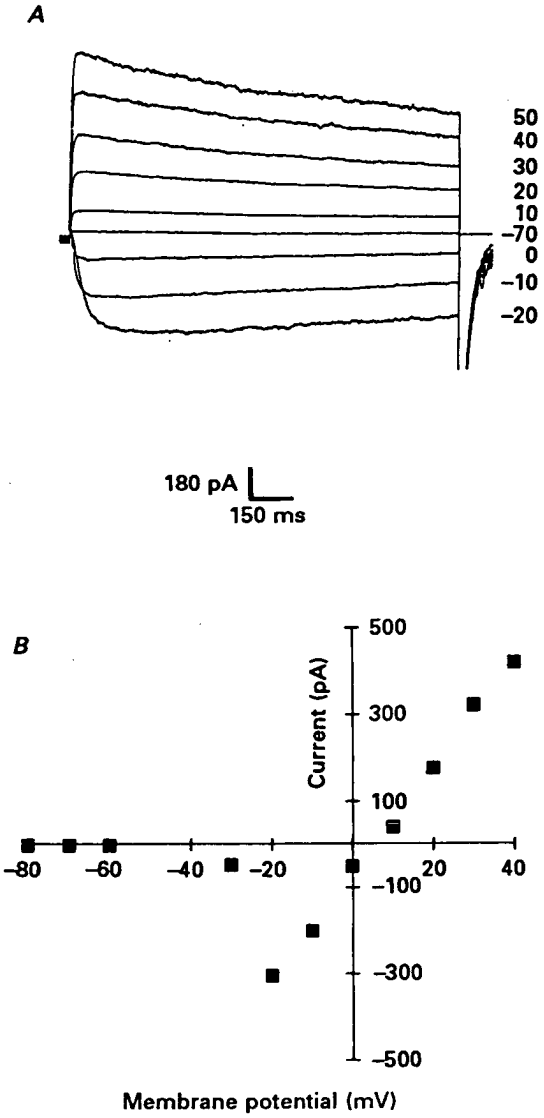

Fig. 1. Global current measured by patch clamp in a Jurkat T cell. *A*, dependency of inward and outward currents on membrane potential. Responses were recorded as a function of depolarizing pulses at −70, −20, −10, 0, 10, 20, 40 and 50 mV. The HP was −80 mV. Depolarizing pulses were applied at 30 s intervals. *B*, current–voltage relationship in Jurkat cells. The current amplitude was measured at the inward and outward peak values.

Outward current

The outward current was studied in cells that showed no inward current. From an HP of −80 mV, the outward current began to be activated for membrane potentials positive to −60 mV. Data shown in Fig. 2*A* indicate that the current was rapidly activated and then was slowly inactivated to a non-zero steady state. The current-

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voltage relationship is shown in Fig. 2*B*. In this case, the amplitude was measured at the peak of the outward current after subtracting the leak current.

Kinetics of activation

Current traces plotted on an expanded time scale showed that the activation phase was sigmoidal and that the rate of activation increased with membrane depolarization (Fig. 3*A*, inset). Kinetic analysis of this current was performed for various

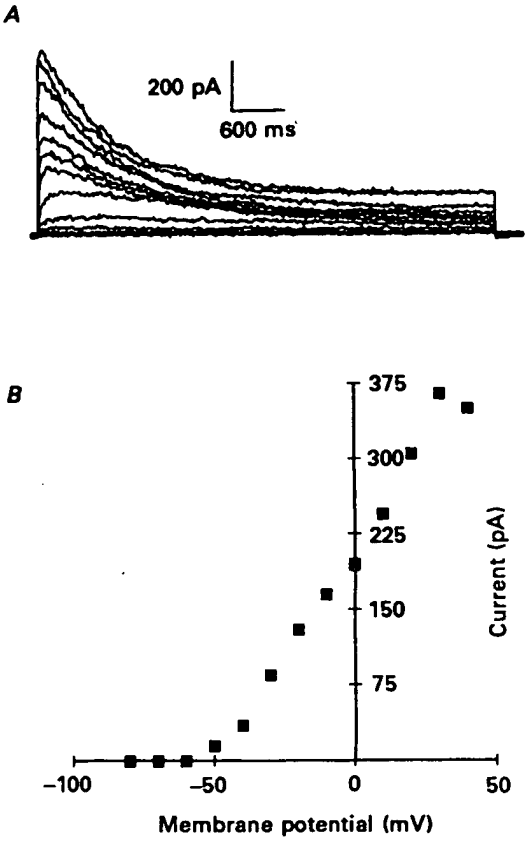

Fig. 2. Outward K^+ current in Jurkat T cells. *A*, time course profile of the K^+ current in a single cell. Depolarizing pulses of 2·8 s duration were applied at 1 min intervals from −80 mV to +40 mV, by 10 mV increments. The current was recorded 10 min after the whole-cell configuration patch was established. *B*, current–voltage relationship for the outward K^+ current, after subtracting the leak current. The HP was −80 mV.

voltage values. The activation phase was fitted using the following equation (Hodgkin & Huxley, 1952),

$$I_t = I_{max}(1-e^{-t/\tau_n})^x, \quad (1)$$

where I_{max} is the maximum K^+ current, τ_n is the activation time constant and, x is the exponent of the activation process. We found that the best fit of the experimental data was for $x = 4$. Figure 3*A* shows results obtained with four different Jurkat cells.

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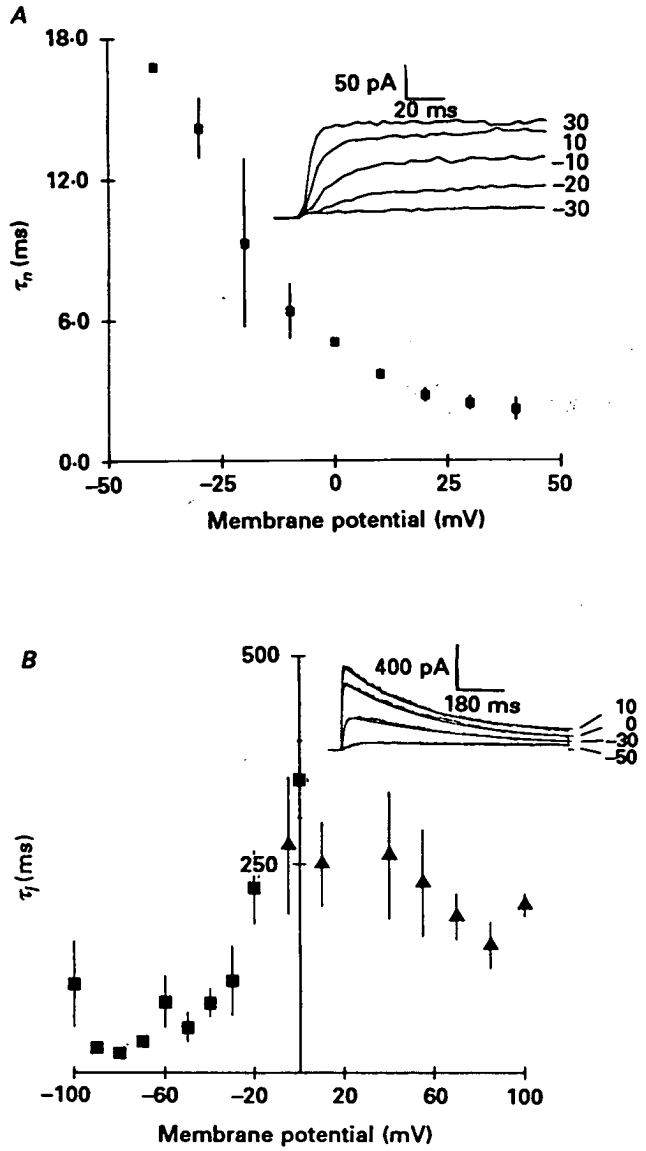

Fig. 3. Kinetics of the outward K^+ current in a Jurkat T lymphocyte. *A*, activation phase: the activation phase is illustrated in the inset. The time constant of the activation phase (τ_n) is plotted as a function of the membrane potential. Each point is the mean of four different experiments. The bars represent S.E.M. *B*, inactivation phase: the inactivation phase was fitted with a single-exponential function. The time constant (τ_j) is plotted as a function of the membrane potential. The values were obtained from the tail current (■) and from the inactivation phase of the current (▲). Each point is the mean of four to five experiments. The bars represent S.E.M. Inset: fitted curves obtained using the following equation,

$$I_t = I_{max}(1 - e^{-t/\tau_n})\, e^{-t/\tau_j} + I_1,$$

where I_{max} is the maximum current at a given potential, τ_n and τ_j are the time constants of the activaction and inactivation process, and I_1 is a time-independent leak current.

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It was found that τ_n was voltage-dependent and decreased from 17 ms at −40 mV to 2 ms at +40 mV.

Kinetics of inactivation

As shown in Fig. 2*A*, the outward current was inactivated on sustained membrane depolarization. The inactivation phase of the current was fitted by a single-exponential function and the inactivation time constant (τ_j) was plotted for various

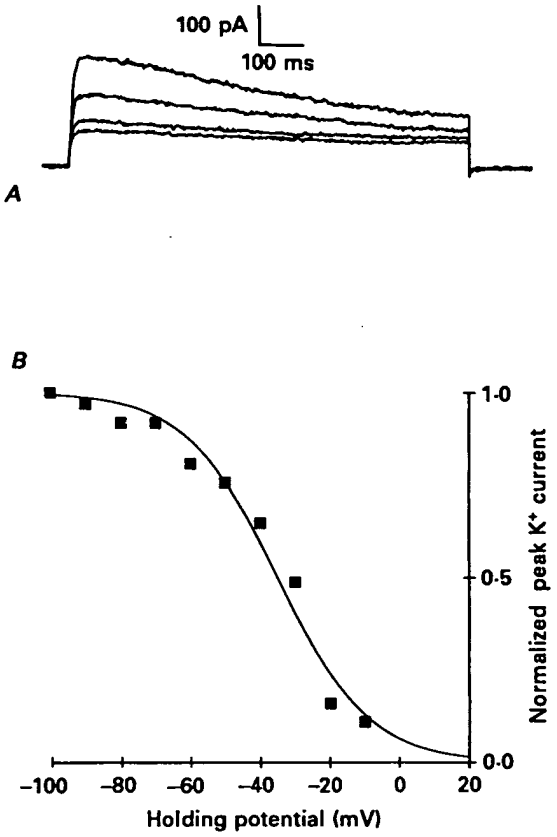

Fig. 4. Inactivation of the outward K^+ current in a Jurkat T cell. *A*, time-dependent accumulation of the current elicited by a depolarizing pulse to 0 mV from an HP of −80 mV. The pulse was applied every 10 s. *B*, voltage dependence of the steady-state inactivation. The current elicited by a pulse to +10 mV was recorded for various HPs that were maintained for 2 min to allow for the equilibrium to be reached. The normalized peak current is plotted as a function of the HP. The continuous line is fitted to the experimental data using eqn (2), where the values for V_k and k are −35 mV and 13 mV, respectively.

membrane potentials. Results from these experiments are shown in Fig. 3*B* (▲). In the case of potentials ranging between −20 and +100 mV, τ_j decreased from 250 ms to 150 ms. The deactivation time constant of the tail current was obtained by stepping the membrane potential from −80 mV to −10 mV with a brief pulse, followed by a test pulse applied at various levels. The decay of the tail current was

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fitted by a single-exponential function. The time constant of the tail current (τ_{tail}) was plotted as a function of the test pulse voltage as shown in Fig. 3*B* (■).

The inactivation of the outward current was measured as a function of time intervals between stimulation or under steady-state conditions. The accumulation of the conductance inactivation was tested by stepping the membrane to +10 mv from an HP of −80 mV, at a frequency of 1 stimulation/10 s. Under these experimental

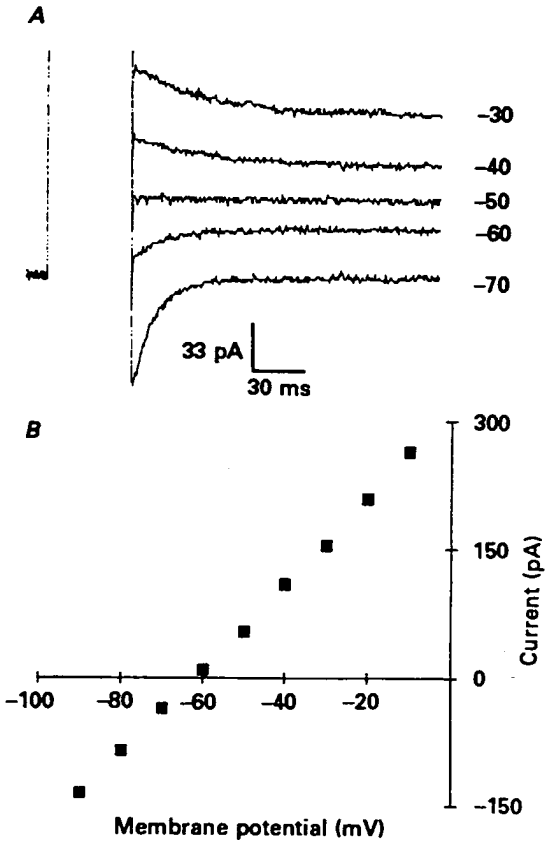

Fig. 5. Zero current voltage of the outward K^+ current in a Jurkat T cell. *A*, the tail currents were obtained by stepping the membrane potential to +20 mV and then repolarizing to different levels. *B*, current–voltage relationship for the tail current. The current amplitude is plotted as a function of the repolarizing potential. The zero current voltage was estimated to be −61 mV.

conditions, the current amplitude decreased rapidly, as shown in Fig. 4*A*. The voltage dependence of the steady-state inactivation of the outward current was determined by varying the HP and recording the current at a constant membrane potential that brought about full current activation. The time interval between each sweep was 2 min. The inactivation–voltage curve obtained under these conditions is shown in Fig. 4*B*. The curve (continuous) was traced according to the Boltzmann equation,

$$j_\infty = 1/[1+\exp(V-V_k)/k], \tag{2}$$

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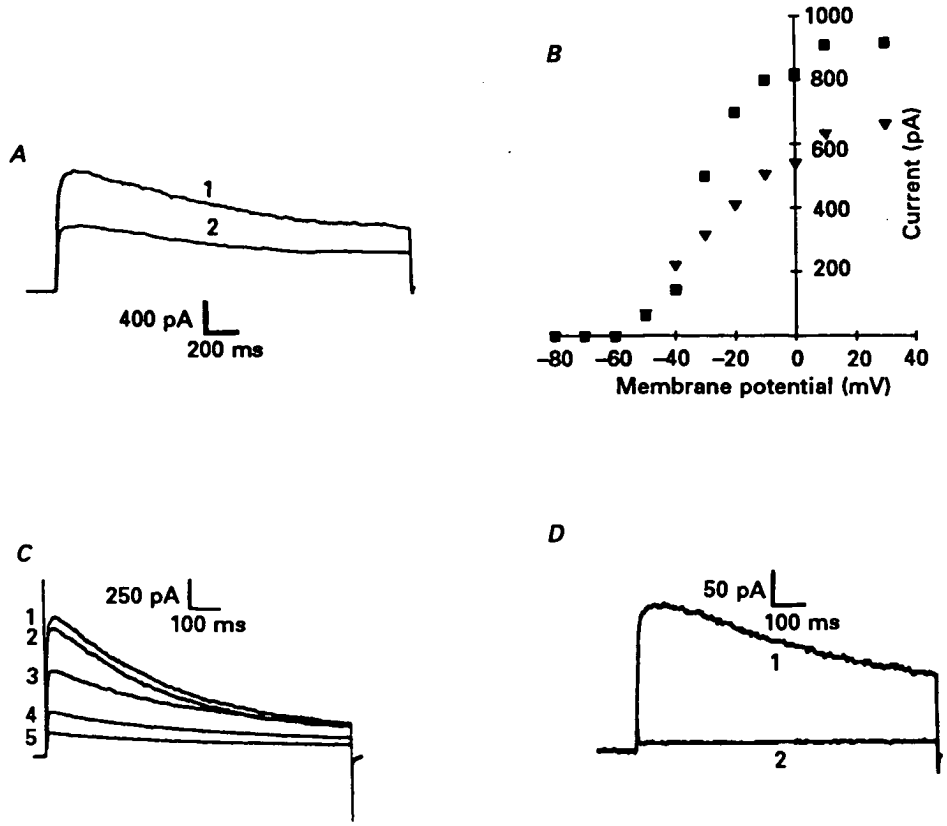

Fig. 6. Effect of the A23187 ionophore on K^+ current in a Jurkat T lymphocyte. *A*, responses to a depolarizing voltage at −10 mV were recorded (1) before and (2) 12 min after the addition of A23187 (1 μM). The HP was −80 mV. *B*, current–voltage relationship of the K^+ current before (■) and after (▼) the addition of A23187 (1 μM). Depolarizing pulses were applied from an HP of −80 mV. *C*, blockage of the outward current by TEA. TEA was added, at various concentrations, to Jurkat cells bathed in GBSS. The current was measured after attaining steady state (5 min). Cells bathed in GBSS (1, control) and in the presence of TEA (mM): 1 (2) 5, (3), 9 (4) and 13 (5). The HP was −80 mV and the membrane potential was −20 mV. *D*, blockage of the outward current 2 min after the addition of Ni^{2+} (1 mM). The HP was −80 mV and the membrane potential was +20 mV.

where V is the membrane potential, V_k is the mid-point potential, and k is the slope factor. The values of the different parameters were obtained by a non-linear least-squares method. Using three different cells, these values were $-32 \pm 2{\cdot}6$ and $10{\cdot}8 \pm 1{\cdot}8$ mV, respectively.

Zero current voltage

The zero current voltage of the outward current was not easily determined from the current-voltage relationship (Fig. 1*B*), and the tail current method was used. Figure 5*A* shows tail currents observed when cells were placed in GBSS containing 5·4 mM-KCl. From an HP of −80 mV, a brief pulse to +10 mV elicited an outward current, after which the cell was plotted as a function of the repolarizing voltage and

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the results are shown in Fig. 5*B*. It was estimated that the zero current voltage had a value of −61 mV. An average of $-61{\cdot}3 \pm 3{\cdot}5$ mV was found ($n = 3$).

Sensitivity to Ca^{2+}

Several groups have reported the sensitivity of K^+ channels to variations in Ca^{2+} concentrations in a number of somatic cells (Meech, 1978). We therefore asked the

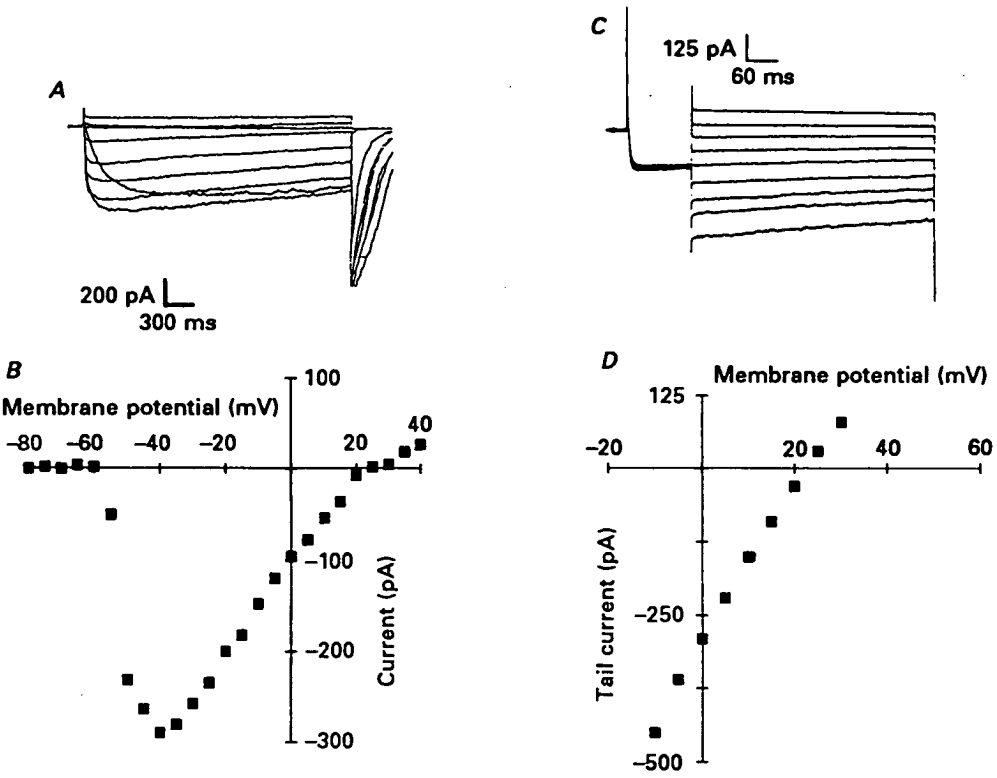

Fig. 7. Slow inward current in Jurkat T cells. The current was recorded with CsCl-filled pipettes in order to block the outward current. The external medium contained 2·2 mM-$CaCl_2$. *A*, current traces obtained for depolarizing pulses from −80 mV to +20 mV, in 10 mV increments. The HP was −80 mV. *B*, current–voltage relationship for the slow inward current. The current starts to be activated at −60 mV and zero current voltage is found at +25 mV. The HP was −80 mV. *C*, tail currents were obtained by stepping the membrane potential to +10 mV and then applying a repolarizing pulse from −10 mV to +30 mV, in 5 mV increments. *D*, current–voltage relationship for the tail current.

question whether this was the case for Jurkat cells. $[Ca^{2+}]_i$ was increased by incubating the cells with the well-known ionophore A23187 and the results are shown in Fig. 6*A*. A significant decrease in the current amplitude was evident after 10 min of incubation. Similar results were obtained with six different patched cells, where the current amplitude was decreased by 31 ± 16%. Current–voltage relationships in the case of cells incubated in the presence (▼) or absence (■) of A23187 are shown in Fig. 6*B*. At 0 mV, the amplitude of the current was decreased by approximately 34%.

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The possibility of a direct effect of A23187 on K^+ current was assessed by incubating the cells in a Ca^{2+}-free medium. In this instance, the current amplitude was not affected or was slightly increased (data not shown).

Channel blocking

Various concentrations of tetraethylammonium (TEA) were used to block the outward current. Figure 6*C* shows the sensitivity of the K^+ current to TEA, after a

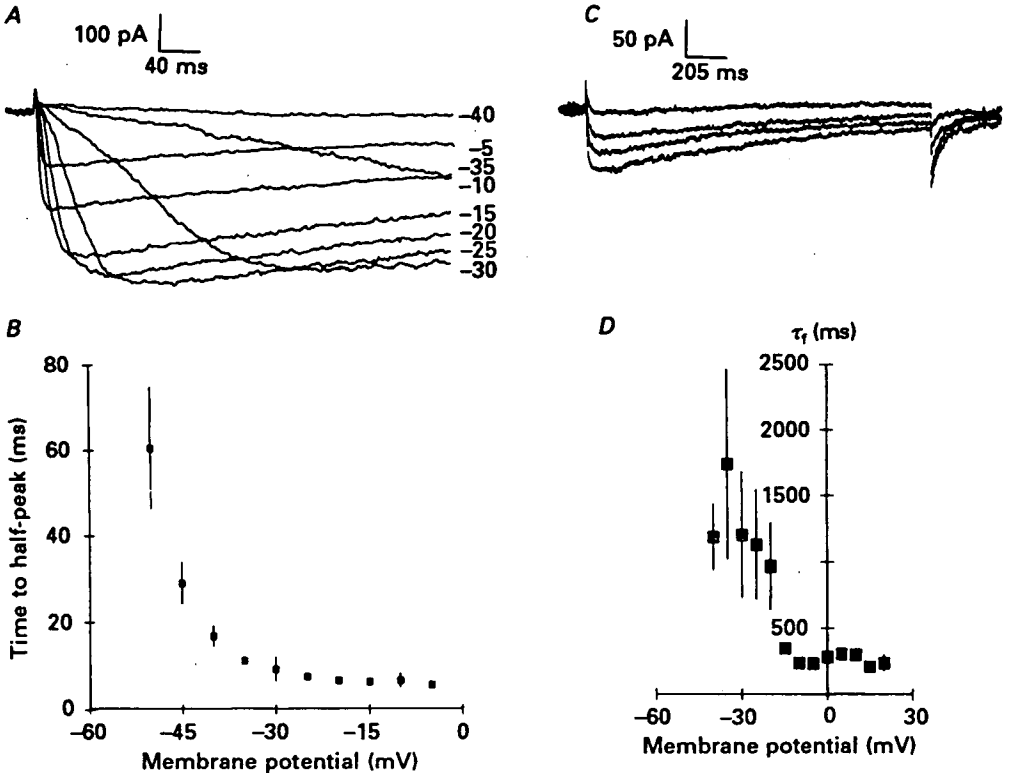

Fig. 8. Kinetics of the slow inward current in a Jurkat T cell. *A*, activation phase of the slow inward current plotted on an expanded time scale. The HP was −80 mV. *B*, time to half-peak plotted as a function of the membrane potential. Values are the mean of four different experiments. The bars represent S.E.M. *C*, inactivation phase of the slow inward current. Membrane potentials were stepped from −40 to −25 mV in 5 mV increments. The HP was −80 mV. *D*, time constant of inactivation (τ_f) plotted as a function of the membrane potential. Values are the mean of six different experiments. The bars represent S.E.M.

5 min perfusion following steady state. In this cell, the TEA concentration required to reach 50% inhibition was 6 mM. Three other experiments gave similar results. In addition, the outward K^+ current was rapidly blocked by incubating Jurkat cells (2 min) in the presence of Ni^{2+} (Fig. 6*D*), a specific Ca^{2+} channel inhibitor (Tsien, Hess, McCleskey & Rosenberg, 1987). Similar results were obtained in the case of other inhibitors such as Co^{2+} ($n = 4$), Mn^{2+} ($n = 3$) or Cd^{2+} ($n = 7$); these data are not shown.

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EXHIBIT B

Inward current

A voltage-dependent inward current was recorded in 40% of Jurkat cells (n = 120), whether outward current was present or inhibited by using Cs^+-loaded pipettes.

Current–voltage relationships and reversal potential

These experiments were carried out on cells incubated in the presence of Ca^{2+} (2·2 mM) and with pipettes filled with CsCl (130 mM) in order to block the outward

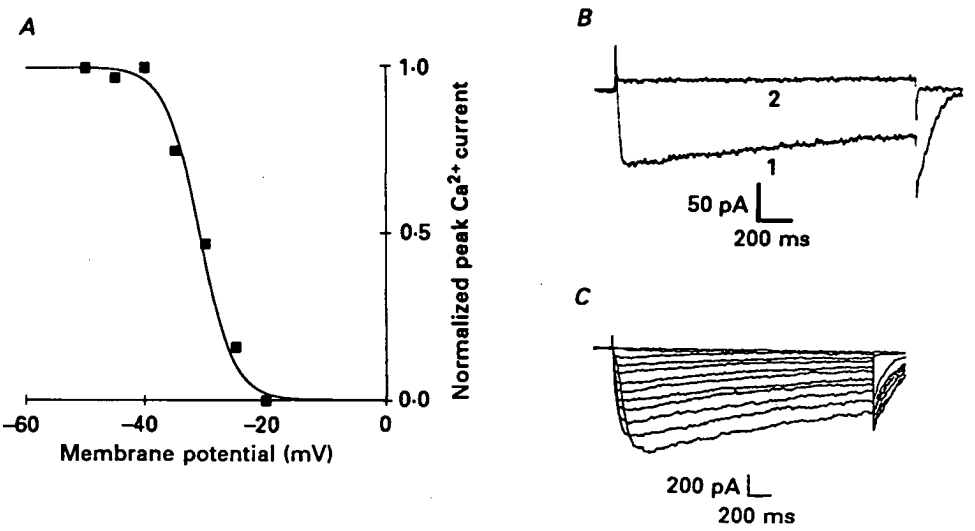

Fig. 9. Voltage dependence of the steady-state inactivation of the inward current in a Jurkat T cell. *A*, the membrane potential was stepped to −10 mV and the HP was varied. The normalized peak currents are plotted *versus* the HPs. The continuous line was drawn according to the Boltzmann function, where $V_k = -31$ mV and $k = 2{\cdot}8$ mV. *B*, blocking effect of Cd^{2+} (1 mM) on the slow inward current. Tracings represent experiments carried out before (1) and 2 min after (2) addition of Cd^{2+}. *C*, currents recorded with cells placed in GBSS containing Sr^{2+} (2·2 mM). Membrane potentials were varied from −80 mV to −10 mV, in 5 mV increments. The HP was −80 mV.

current. A family of current traces obtained for various depolarizations from an HP of −80 mV is shown in Fig. 7*A*. Tracings show an obvious rapid current activation and a slow inactivation that is a function of time. The current–voltage relationship shows that the threshold voltage was near −60 mV, whereas the peak of the maximum current was found at approximately −40 mV and the zero current voltage was positive to +20 mV (Fig. 7*B*).

The tail current method was used to determine the zero current voltage, as described above in the case of the outward current. Results are shown in Fig. 7*C*. The amplitude of the tail current was plotted as a function of the repolarizing voltage and had a value of zero at +23·6 mV (Fig.7*D*). Six similar experiments gave a mean value of 19·0±4·1 mV.

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Kinetics of the inward current

Figure 8*A* shows current traces on an expanded time scale. In the case of negative membrane potentials, the current activates very slowly and activation becomes faster with depolarization. An estimate of the activation rate was obtained by determining the time needed to reach 50% of the maximum amplitude (rise time to half-maximum current, $t_{\frac{1}{2}}$) at a given membrane potential (Cahalan *et al.* 1985). The results are shown in Fig. 8*B* ($n = 4$). When the membrane potential was negative (−50 mV), $t_{\frac{1}{2}}$ was 60 ms and grew shorter with increasing depolarization. For instance, at −5 mV $t_{\frac{1}{2}}$ was 5 ms.

Inactivation of the inward current was very slow and was completed only after approximately 2–3 s, as shown in Fig. 8*C*. The inactivation phase of the current was fitted with a single-exponential function and the inactivation time constant τ_f was plotted as a function of the membrane potential (Fig. 8*D*).

Steady-state inactivation

Steady-state inactivation experiments were performed by changing the HP and measuring the inward current elicited by stepping the membrane to −10 mV. For each HP studied, there was a waiting period of 2 min to allow the system to reach equilibrium. The normalized peak currents were plotted against HP values and the results are shown in Fig. 9*A*. The curve (continuous) was drawn according to the Boltzmann equation, using the parameters $V_k = -31$ mV and $k = 2{\cdot}84$ mV. From three experiments we found that V_k had a value of $-31 \pm 3{\cdot}4$ mV and k had a value of $3{\cdot}3 \pm 0{\cdot}2$ mV.

Channel blockers and selectivity

Inorganic ions are known to block the inward Ca^{2+} current in a variety of cells (Tsien *et al.* 1987). In the case of Jurkat cells, we observed that Co^{2+} (3 mM) and Mn^{2+} (3 mM) completely inhibited the inward current after 2–3 min of incubation (data not shown). In Fig. 9*B* we present data on the inhibition of the inward current by Cd^{2+} (1 mM). Clearly, the inward current was totally inhibited after a 2 min incubation period.

It is well established that Ca^{2+} channels are permeable to divalent cations such as Sr^{2+} (Tsien *et al.* 1987). Substitution of Ca^{2+} for an equimolar concentrations of Sr^{2+} did not change the kinetics or the amplitude of the inward current in Jurkat cells (Fig. 9*C*). These observations suggest that, in the case of Jurkat cells, Ca^{2+} channels appear to be equally permeable to Ca^{2+} and Sr^{2+} ions.

Effects of PHA on the inward and the outward currents

Since we had observed that the outward current amplitude decreased when $[Ca^{2+}]_i$ was augmented (Fig. 6) and since PHA induces increased $[Ca^{2+}]_i$ (Weiss *et al.* 1986) in Jurkat cells, we investigated the possibility that the effect of the lectin on the outward current amplitude may involve Ca^{2+} channels. Experiments were performed on the global ionic current and results are shown in Fig. 10. In the case of control experiments (absence of PHA, cells in GBSS containing 2·2 mM-Ca^{2+}) a membrane

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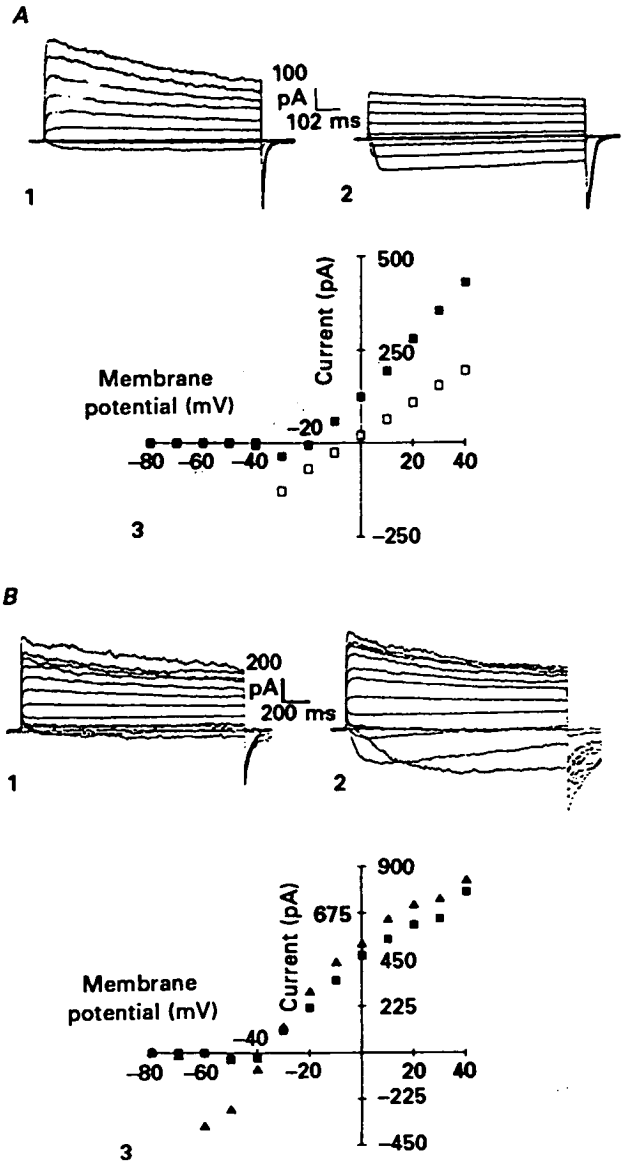

Fig. 10. Representative examples of the effects of PHA on the inward and outward currents of Jurkat T cells. *A*, effect of PHA on the global current. Jurkat T cells were bathed in GBSS medium containing Ca^{2+} (2·2 mM): (1) current traces obtained under control (absence of PHA) conditions. Membrane potentials were varied from −40 mV to +30 mV, in 10 mV increments. The HP was −80 mV, (2) currents recorded in the same cell after addition of PHA (10 μg/ml), (3) current–voltage relationships for the global current before (■) and after (□) the addition of PHA (10 μg/ml). *B*, effect of PHA on the global current of a Jurkat T lymphocyte bathed in GBSS containiung Sr^{2+} (2·2 mM): (1) current traces obtained under control (absence of lectin) conditions. Membrane potential was varied from −80 mV to +30 mV, in 10 mV increments. The HP was −80 mV, (2) currents recorded in the same cell after the addition of PHA (10 μg/ml), (3) current–voltage relationships for the global current before (■) and after (▲) the addition of PHA (10 μg/ml).

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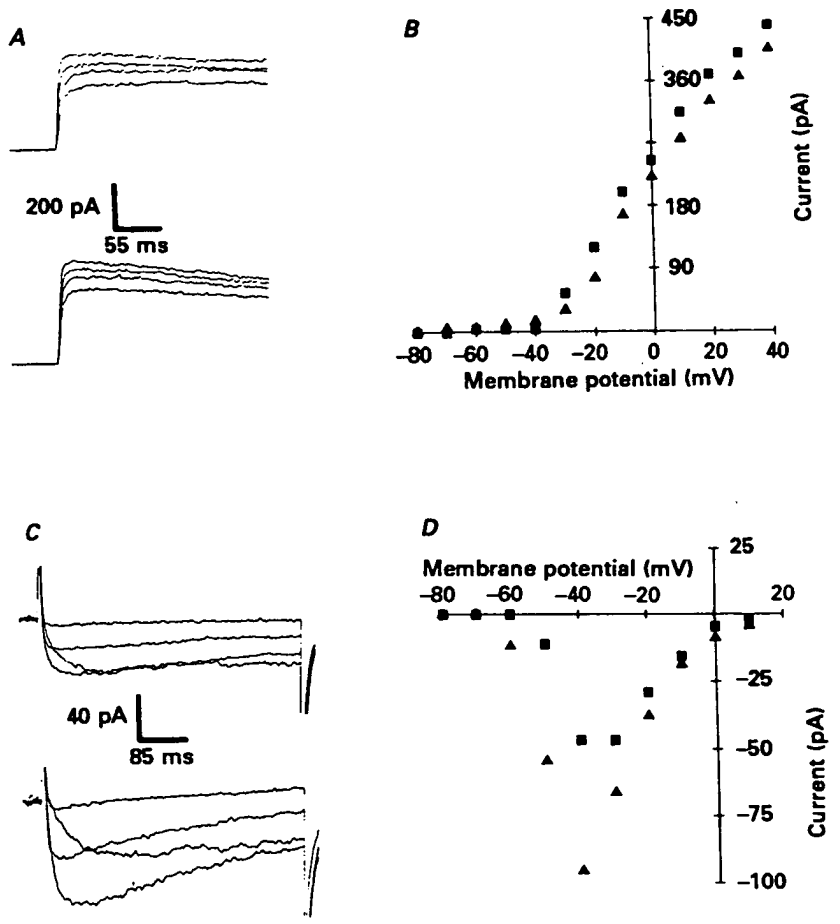

Fig. 11. Effects of PHA on the outward current in a Jurkat cell. *A*, the outward current was recorded in a Jurkat T cell that did not display any inward current. Data are presented for the control (absence of PHA) and experimental conditions (PHA, 10 μg/ml). The HP was −80 mV, and membrane potentials were 10, 20, 30 and 40 mV, respectively. *B*, current–voltage relationships before (▲) and after (■) addition of PHA (10 μg/ml). Effects of PHA on the inward current in a Jurkat cell. *C*, experiments were carried out under conditions in which the outward current was blocked (CsCl in the patch electrode). The upper part of the panel represents control conditions (absence of PHA) and the lower part experimental conditions (PHA, 10 μg/ml). The HP was −80 mV and the membrane was depolarized to −50, −40, −30 and −20 mV. *D*, current–voltage relationships before (■) and after (▲) addition of PHA (10 μg/ml). The leak current was subtracted.

potential positive to −50 mV elicited inward currents from an HP of −80 mV (Fig. 10*A*1). When the potential was more positive than −20 mV, outward currents were recorded, as shown in Fig. 10*A*. Addition of PHA (10 μg/ml) induced two obvious major changes in the current trace pattern: an increase in the inward current amplitude and a decrease in the outward current amplitude (Fig. 10*A*2). These changes are summarized in Fig. 10*A*3 where the respective current–voltage relationships are illustrated. In studies with eight different cells, the outward current was decreased by 45 ± 12 %.

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Similar experiments using a Ca^{2+}-free, Sr^{2+}-containing medium showed that the Sr^{2+} current was increased by the lectin, while the outward current was not affected (Fig. 10*B*1 and *B*2). The current–voltage relationship showed an increase in the range of voltage where the current was inward, but no changes were detected in the outward portion of the curve (Fig. 10*B*3).

Moreover, the effect of PHA was assessed in cells which did not display an inward current. In these cases ($n = 5$), the outward current was increased by $17{\cdot}6 \pm 13\%$ at -20 mV (Fig. 11*A* and *B*). The inactivation time constant τ_j decreased for all of the voltage ranges studied, with specific decreases of $-38{\cdot}5 \pm 9$ and $-23 \pm 4\%$ at 0 and $+40$ mV, respectively.

Figure 11*C* shows the current traces obtained in patched Jurkat cells at various membrane potentials from an HP of -80 mV. In this case, the external GBSS medium contained 2·2 mM-$CaCl_2$ and pipettes were filled with CsCl (120 mM). After addition of PHA (10 μg/ml), the amplitude of the current increased significantly as shown in Fig. 11*C*. The current–voltage relationship is shown in Fig. 11*D*. Kinetic analysis of the inactivation phase of the Ca^{2+} current showed that PHA had no effect on τ_f ($n = 4$, data not shown).

DISCUSSION

The present study demonstrates the presence, in the Jurkat T cell line, of an outward K^+ current as well as an inward Ca^{2+} current that shows time- and voltage-dependent kinetics.

K^+ channels

The outward K^+ current presents activation and inactivation kinetics similar to those previously observed for human peripheral T lymphocytes (DeCoursey *et al.* 1984; Matteson & Deutsch, 1984; Cahalan *et al.* 1985; Bregestovski, Redkozubov & Alexeev, 1986), murine T cells (DeCoursey, Chandy, Gupta & Cahalan, 1987) and murine cytotoxic (Fukushima *et al.* 1984) and helper (Lee, Sabath, Deutsch & Prystowsky, 1986) T lymphocytes.

K^+ channels activated by $[Ca^{2+}]_i$ have been found in neurones and in many excitable cells (Meech, 1978). However, some types of K^+ channels such as those found in human T lymphocytes (Bregestovski *et al.* 1986) or in murine B cells (Choquet, Sarthou, Primi, Cazenaven & Korn, 1987) are blocked by an increase in $[Ca^{2+}]_i$. This also appears to be the case for Jurkat cells as evidenced by the effect of the calcium ionophore A23187 (Fig. 6) or ionomycin (not shown). PHA also reduces the outward K^+ current with a concomitant increase of the Ca^{2+} current (Fig. 11). In contrast, the lectin increased the Sr^{2+} current but this did not affect the K^+ current. We thus postulate that the PHA-dependent decreased K^+ current observed in Jurkat cells is due to a lectin-dependent increase in $[Ca^{2+}]_i$. This proposition is based on (1) the fact that PHA increases $[Ca^{2+}]_i$ in Jurkat cells (Weiss *et al.* 1986), (2) an observable increase in Ca^{2+} current in these cells (Fig. 11), (3) the effect of ionophores on the K+ current (Fig. 6) and (4) the lack of effect of PHA on cells that did not display any inward current. In keeping with this last result, we have found that PHA increased the outward current in cells that did not display an inward current

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(Fig. 10*A* and *B*). These observations are in agreement with those of Cahalan *et al.* (1985) who reported that PHA increased the amplitude of the peak conductance by altering the external surface potential (DeCoursey *et al.* 1984). However, Schlichter, Sidell & Hagiwara (1986) have suggested that this phenomenon is not an essential event in the T cell response to PHA.

The potency of TEA to block the delayed outward K^+ current in somatic cells depends on the type of K^+ channels involved. For instance, the concentration needed for 50% inhibition has been found to be 8–16 mM in the case of type n K^+ channels, whereas it is 50–100 μM in the case of type l K^+ channels (DeCoursey *et al.* 1987). In the present study, our data suggest that Jurkat cells posesss type n K^+ channels.

Ca^{2+} channels

The existence of Ca^{2+} channels in lymphoid cells was originally proposed by Michell (1975), but the presence of such channels could not be recorded in human (Matteson & Deutsch, 1984; Cahalan *et al.* 1985) or in cloned murine T lymphocytes (Fukushima *et al.* 1984). However, macroscopic as well as unitary voltage-independent Ba^{2+} currents have been observed in cloned human helper T lymphocytes (Kuno *et al.* 1986) and unitary Ca^{2+} currents have been reported in Jurkat cells (Kuno & Gardner, 1987) and in T lymphocyte tumour cell markers reconstituted in bilayer phospholipid membranes (Pecht, Corcia, Liuzzi, Alcover & Reinherz, 1987).

Our results show conclusively the presence of voltage-dependent Ca^{2+} channels in approximately 40% of the Jurkat cells studied. It is of note to mention that MacCumber & Tucker (1987) have reported that 50% of peripheral human lymphocytes respond to PHA by increasing $[Ca^{2+}]_i$. This observation may be related to the frequency of Ca^{2+} currents observed in the present study.

At a holding potential of -80 mV and in the presence of external Ca^{2+} (2·2 mM), the current starts to be activated at a voltage positive to -60 mV, the maximum current is found near -40 mV and the zero current voltage is observed between $+20$ and $+30$ mV. This low value for the zero current voltage could be due to an outward current of monovalent cations (Cs^+) flowing through the Ca^{2+} channels, as reported by Fukushima & Hagiwara (1985).

Recently, various types of Ca^{2+} channels have been described and they differ in voltage thresholds, steady-state inactivation curves, kinetics and response to pharmacological agents (Nowycky, Fox & Tsien, 1985). Although the current found in Jurkat T cells shows a slow kinetic pattern similar to that of the long-lasting (L-type) channel, the threshold voltage was more negative than that of a typical L-type channel. Further work using channel agonists or antagonists is obviously required to classify the Ca^{2+} channel observed in Jurkat cells.

The observation that a voltage-dependent Ca^{2+} is present in the leukemic Jurkat T cell line whereas it has not been previously reported in peripheral blood T lymphocytes, may suggest that calcium channels are too small in number to be detected as macroscopic current in normal T cells. In addition, cell transformation may be accompanied by an increase in the number of Ca^{2+} channels (Fukushima & Hagiwara, 1983).

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Effects of PHA on ionic currents in Jurkat cells

PHA is known to stimulate T lymphocyte from various species to undergo blast transformation and mitosis (Gelfand *et al.* 1987). One of the earliest events that is associated with PHA-dependent lymphocyte stimulation is an increase in $[Ca^{2+}]_i$ and a depolarization of the plasma membrane (Gelfand *et al.* 1987).

With respect to changes in $[Ca^{2+}]_i$, Tsien *et al.* (1982) initially reported that PHA brings about an approximately twofold increase in $[Ca^{2+}]_i$ in porcine lymphocytes. Similar results have been reported by Gelfand, Cheung & Grinstein (1986) in the case of human peripheral T cells and by Weiss *et al.* (1986) in Jurkat T cells. The source of Ca^{2+} involved in such increases has been established. The largest contribution comes from extracellular Ca^{2+} that is responsible for the sustained increase in $[Ca^{2+}]_i$ (Oettgen, Terhorst, Cantley & Rosoff, 1985; Alcover *et al.* 1987; Imboden & Weiss, 1987), whereas, Ca^{2+} mobilization from intracellular stores, probably as a result of inositol 1,4,5-trisphosphate ($InsP_3$) release, accounts for the early rise in $[Ca^{2+}]_i$ (Berridge & Irvine, 1984). It has been postulated that lymphocyte Ca^{2+} channels involved in the cytosolic increase of this ion may be regulated directly by the T cell antigen–receptor complex (Oettgen *et al.* 1985) or indirectly by $InsP_3$ (Kuno & Gardner, 1987). Alternatively, Cahalan *et al.* (1985) have suggested that Ca^{2+} entry into lymphocytes could proceed via K^+ channels. However, reports by Gelfand *et al.* (1986) and data presented in this study do not agree with this hypothesis (Fig. 10). Our observations suggest that cytosolic increases in Ca^{2+} concentration previously observed in Jurkat cells (Weiss *et al.* 1986) may, at least in part, be mediated via voltage-dependent calcium channels. Whether PHA acts directly on such channels or whether the action is mediated through a second messenger such as $InsP_3$ (Kuno & Gardner, 1987) remains to be determined.

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POTASSIUM AND CALCIUM CHANNELS IN LYMPHOCYTES

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ABSTRACT

Over the past decade, a variety of ion channels have been identified and characterized in lymphocytes by use of the patch-clamp technique. This review discusses biophysical and regulatory aspects of lymphocyte potassium and calcium channels with the aim of understanding the role of these channels in lymphocyte functions. Lymphocytes express both voltage-dependent potassium [K(V)] channels and calcium-activated potassium [K(Ca)] channels, and each is upregulated as cells progress toward division following mitogenic stimulation. The genes encoding two K(V) channels, Kv1.3 (type *n*) and Kv3.1 (type *l*), have been cloned. Mutational analysis is revealing functionally important regions of these channel proteins. Exogenous expression studies and the use of highly specific channel blockers have helped to establish the roles of type *n* K(V) channels in sustaining the resting membrane potential, in regulating cell volume, and in enabling lymphocyte activation. Blockade of K(V) and K(Ca) channels effectively inhibits the antigen-driven activation of lymphocytes, probably by inducing membrane depolarization and thereby diminishing calcium influx. A prolonged rise in intracellular calcium ($[Ca^{2+}]_i$) is a required signal for lymphocyte activation by antigen or mitogens. Single-cell fluorescence measurements have revealed underlying $[Ca^{2+}]_i$ oscillations that are linked closely to the opening and closing of Ca^{2+} and K^+ channels. Sustained Ca^{2+} signaling and oscillations depend absolutely on plasma-membrane Ca^{2+} channels that are activated by the depletion of intracellular calcium stores. Under physiological conditions these channels

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open as a consequence of store depletion induced by inositol 1,4,5-trisphosphate (IP_3), but they can also be activated experimentally by several agents that empty the stores without generating IP_3, such as the microsomal Ca^{2+}-ATPase inhibitor thapsigargin. The intricate causal relationships among ion channels, membrane potential, $[Ca^{2+}]_i$, and lymphokine gene expression can now be pursued at the single-cell level with patch-clamp recording, calcium-dependent dyes, reporter genes, and fluorescence video techniques. These approaches will help to clarify the essential roles of ion channels in the molecular pathways subserving activation and other lymphocyte behaviors.

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INTRODUCTION

Ion channels and fluxes have long been suspected to play a role in lymphocyte signal transduction, but only relatively recently have specific channel types been defined at biophysical or molecular levels. Unlike nerve and muscle cells, lymphocytes lack electrical excitability, and yet patch-clamp studies have revealed a surprisingly complex biophysical phenotype, with multiple K^+, Ca^{2+}, and Cl^- channels expressed in patterns that are regulated differentially according to cell subset and state of activation. By controlling ion fluxes across the plasma membrane, channels mediate changes in intracellular ion concentrations and membrane potential in response to a variety of stimuli. The properties and possible functions of many of these channels have been reviewed (1–10). This review focuses on recent developments in understanding the properties and functional roles of K^+ and Ca^{2+} channels in lymphocytes. Substantial progress has been made in this area by the application of molecular biological and patch-clamp techniques together with single-cell fluorescence measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and reporter gene expression. We specifically address several outstanding issues concerning the functional roles of channels in lymphocytes, including 1) the requirement of K^+ channels for T cell activation; 2) the identity of the mitogen-stimulated Ca^{2+} channel and its mode of regulation; and 3) the means by which K^+ channels and Ca^{2+} channels may interact to influence cell-activation events.

POTASSIUM CHANNELS IN LYMPHOCYTES

Molecular Cloning of Voltage-Gated K^+ Channels in Lymphocytes

Several types of voltage-gated K^+ [K(V)] channels have been intensively studied in lymphocytes through patch-clamp studies of a variety of immature and mature T cells, B cells, and lymphoid cell lines. For convenience, these have been named types *n* (for prevalence in *normal* human T cells) (11, 12), *n'* (similar to type *n*) (13), and *l* (*large* conductance and abundance in T cells from autoimmune *lpr* mice) (14). The three channel types share two fundamental

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properties: they are opened by membrane depolarization, and they are K^+-selective. However, they differ in their voltage sensitivity, their tendency to inactivate during prolonged depolarization, their kinetic behavior, their single-channel conductance, and their sensitivities to a range of pharmacological agents. The distinguishing features of these channels have been reviewed previously in detail (7, 8); the characteristics most relevant to this review are summarized in Table 1.

The molecular cloning of ion-channel genes from different cell types and species has revealed an ever-increasing complexity of the K(V) channel superfamily. Sequence analysis provides a convenient and logical means of grouping the channels into families according to their similarity to several K^+-channel genes originally identified in *Drosophila*: *Shaker* (Kv1), *Shab* (Kv2), *Shaw* (Kv3), and *Shal* (Kv4) (for reviews, see 8, 15). Below we summarize the results of cloning and expression studies that have identified the genes encoding the type *n* (Kv1.3) and *l* (Kv3.1) channels in lymphocytes (for review, see 9). These results establish a basis for identifying residues that are

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Table 1 Potassium and calcium channels in lymphocytes[a]

Name	Conductance (p^S)	Activation (midpoint)	Blockers	Expression (resting)	Expression (proliferating)
K(V) *n* (Kv1.3)	10–18	voltage (−40 mV)	TEA (mM) CTX, NTX, MTX, KTX (nM)	human T (++) mouse T, B (+)	human T (+++) mouse T, B (+++) Jurkat (++)
K (V) *n'*	18	voltage (−30 mV)	TEA (mM) CTX (nM)	mouse T (++)	
K(V) *l* (Kv 3.1)	27	voltage (0 mV)	TEA (μM)	mouse T (++)	Louckes (++)
K(Ca) mini	2–8	Ca^{2+}_i (400 nM)	TEA (mM) apamin (pM)	mouse T (?)	Jurkat (+++)
K(Ca) midi	11–35	Ca^{2+}_i (300 nM)	TEA (mM) CTX (nM)	human T (+) mouse B (+)	human T (+++) mouse B (++) Jurkat (+)
Ca^{2+}	0.01–0.03	Ca^{2+} store depletion	Ni^{2+} (mM) econazole, SKF 96365 (μM)	human T (+++)	Jurkat (+++)

[a]Summary of characteristics, including the channel gene (if known), single-channel conductance, mode of activation, selected pharmacological blockers, and expression levels in resting and proliferating cells. Approximate potencies of blockers are indicated in ranges from mM to pM. Expression levels are approximate: +, 5–50 channels per cell; ++, 100–500 per cell; +++, > 500 per cell; and ?, expression level unknown. See text for references and abbreviations.

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critical in determining biophysical properties and pharmacological profiles. For clarity, the terms *n* and *l* are used below to refer to studies of native channels, while the Kv nomenclature is used in reference to exogenously expressed K^+ channels.

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Kv1.3 ENCODES THE *n*-TYPE CHANNEL IN LYMPHOCYTES Genes encoding the *n* channel were isolated by low-stringency screening of rat or mouse genomic DNA libraries with probes derived from homospecific homologs of the *Shaker* gene (Kv1.1) (16, 17), or by screening a human Jurkat T cell cDNA library with a probe made from the rat brain *Shaker* homolog, RCK1 (18). The resulting clones, classified as Kv1.3, are all very similar. Northern analysis shows that the Kv1.3 transcript is highly expressed in rat thymus (16), and the polymerase chain reaction (PCR) has been used to detect Kv1.3 mRNA in the EL4 T cell line (19). Furthermore, mRNA derived in vitro from the rat, mouse, or human Kv1.3 genes, when expressed in *Xenopus* oocytes, encodes K^+ channels whose biophysical and pharmacological signature closely resembles that of native type *n* channels in lymphocytes (16, 18–20). Similar results were obtained after transient transfection of CTLL-2 cells (a murine cytotoxic T cell line that does not normally express either detectable K^+ currents or Kv1.3 mRNA) with a Kv1.3-containing plasmid (21). Their voltage dependence, rates of opening and closing, single-channel conductance, inactivation properties, and blockade by different compounds including tetraethylammonium (TEA) and charybdotoxin (CTX) were indistinguishable from native channels (Table 1).

Site-directed mutagenesis of the Kv1.3 gene provides a powerful tool to explore structure-function relationships for the type *n* channel. Thus far, the channel's pharmacological sensitivity and inactivation behavior have been studied most extensively. A relationship between the sites that govern blockade by TEA and inactivation was first inferred from the ability of partially blocking doses of TEA to slow the rate of inactivation (22). Similarly, protonation of his401 in the Kv1.3 channel decreases both TEA sensitivity (23) and the inactivation rate (24), and mutation of this histidine to a tyrosine reduces the extent of inactivation approximately fivefold (24). These results suggest that a histidine located near the outer pore region determines how the *n* channel inactivates and binds TEA. One speculation is that each of the four channel subunits contributes a histidine to create a Ca^{2+} binding site (24), which is consistent with biophysical evidence that Ca^{2+} binds to a site near the extracellular mouth of the *n* channel pore to cause or enhance inactivation (25).

Kv3.1 ENCODES THE TYPE *l* K^+ CHANNEL The *Shaw*-related Kv3.1 gene was first cloned by homology from rat and mouse libraries (26, 27). Heterologous expression in oocytes produced a K^+ current with similarities to type *l* channels in terms of the voltage dependence of opening, single-channel conductance, sensitivity to TEA, and insensitivity to CTX (26, 27; see Table 1). Further

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studies showed that the kinetics of inactivation and deactivation (channel closing following the end of a depolarizing stimulus) were also indistinguishable from native *l* channels (28). The extremely close similarities between native *l* channels and Kv3.1 expressed in oocytes suggest that the *l* channel exists in lymphocytes as a homomultimer of four identical Kv3.1 subunits rather than as a heteromultimer containing another Kv3 subfamily member (28). Kv3.1 has also been expressed through transient transfection of CTLL-2 cells, in which it produces a conductance with *l*-type characteristics (21). Finally, Kv3.1 coding sequences have been detected using PCR in two cell types that express functional type *l* K^+ channels: a human B-lymphoma line, Louckes, and $CD4^-CD8^-$ T cells from *lpr* mice (28).

ADDITIONAL VOLTAGE-GATED K^+ CHANNELS IN LYMPHOCYTES Electrophysiological and molecular biological evidence exists for additional K(V) channels in T cells, but these are less abundant than *n*- and *l*-type channels and hence have not received as much attention. A channel classified as type *n'*, similar to type *n* but non-inactivating, is expressed by murine $CD4^-CD8^+$ thymocytes (13, 29); its molecular basis is not yet understood (Table 1). Indirect evidence for a CTX-insensitive K^+ channel in rat thymocytes is based on the inability of CTX to fully inhibit the regulatory volume decrease (RVD) induced by hypoosmotic solutions, a process that requires K^+ efflux and is completely inhibited by less selective K^+-channel blockers (30; for reviews, see 1, 31, 32). Interestingly, one study of single K^+ channels in membrane patches revealed at least two types of K^+ channels in human T cells that appear to be distinct from types *n* and *l* (33), one of which has properties that could explain the CTX-insensitive RVD activity observed in rat thymocytes (30). Several hypotheses have been advanced to explain the molecular origins of additional K^+ channels in lymphocytes, including alternative splicing (although alternative splicing of mammalian Kv1.3 mRNA is not believed to occur—17), expression of additional K^+ channel genes such as IsK (18), heteromultimer formation, and modulation of channel properties by another channel subunit (34), by channel clustering (35, but see 9), or by posttranslational modifications.

Acute Mechanisms of K(V) Channel Modulation

The functioning of K(V) channels in lymphocytes is subject to both acute and long-term modulation. Acute mechanisms are of particular interest, as physiological stimuli including antigen, neurotransmitters, and hormones act through lymphocyte receptors both to generate increases in second messengers such as Ca^{2+} and cAMP and to activate protein kinases (36–39). Results thus far have varied widely among different groups, due in part to the use of different lymphocyte subtypes and protocols, and to the fact that the behavior of K(V) channels is strongly influenced by such experimental factors as temperature and intracellular dialysis during whole-cell recording (11, 12, 40–42). Studies

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of modulation using cloned and modified Kv1.3 gene products may ultimately provide a more uniform basis for comparisons.

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EFFECTS OF CALCIUM There is widespread agreement that intracellular Ca^{2+} exerts an inhibitory effect on *n*-type K^+ channels in both T and B lymphocytes, although multiple mechanisms appear to exist. Bregestovski et al (43) were the first to describe Ca^{2+}-dependent inhibition of K^+ channels in excised patches and in whole-cell recordings from human T cells. Similar inhibition has been observed in cell-attached patch recordings from intact T cells treated with the Ca^{2+} ionophore ionomycin, demonstrating that the effect does not require the dilution of cellular constituents that occurs during excised-patch and whole-cell experiments (44). The *n*-type K^+ current in murine B cells and their precursors is inhibited by micromolar levels of Ca^{2+} in the recording pipette, primarily through a several-fold increase in the rate of inactivation (45). A subsequent detailed study of divalent ion interactions with the type *n* K^+ channel in Jurkat T cells found comparable effects and concluded that Ca^{2+} causes or accelerates inactivation by binding to a site located within the channel pore (25). Taken together, these results suggest that the inhibition by Ca^{2+} is unlikely to involve a kinase, phosphatase, or other diffusible cytosolic molecule.

A different type of Ca^{2+}-dependent inhibition has been observed in oocytes coinjected with mRNA for Kv1.3 and 5-HT_2 receptors (46–48). In these cells, 5-HT elicits a rise in $[Ca^{2+}]_i$ through the activation of phospholipase C (PLC) and production of inositol 1,4,5-trisphosphate (IP_3), and inhibits the K^+ current by 70–80%. Similar results were produced by directly injecting IP_3 or Ca^{2+}, and the 5-HT effect was slowed or blocked by injection of Ca^{2+} chelators, suggesting that it is mediated through a rise in $[Ca^{2+}]_i$. However, unlike the Ca^{2+}-dependent inhibition described above for lymphocytes, 5-HT-induced inhibition in oocytes is nearly irreversible and does not involve a change in the rate of channel inactivation. A similar long-lasting inhibition of type *n* channels by Ca^{2+} has not been reported in lymphocytes, although 5-HT binding to 5-HT_1 and 5-HT_3 receptors increases the maximum K^+ conductance and accelerates inactivation in a pre-B cell line (49). The different effects of Ca^{2+} on K^+ channels in oocytes as opposed to lymphocytes may reflect differences in the biochemical microenvironment or in the expression of auxiliary channel subunits. In this regard, a 41-kDa β subunit of Kv1 channels has been described that appears to play an important role in the inactivation process (50), and a protein of this size is associated with the type *n* channel in T cells (34).

EFFECTS OF PHORBOL ESTERS Phorbol esters, potent activators of protein kinase C (PKC), lack acute effects on type *n* K^+ current in human peripheral blood T cells (12), but they do appear to inhibit *n* channels in Jurkat T cells (51) and Kv1.3 channels expressed in oocytes (47, 48). The primary sequence of Kv1.3 contains a highly conserved intracellular consensus site for PKC

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phosphorylation (34). A biochemical study has shown that the native type *n* channel from unstimulated Jurkat cells is phosphorylated exclusively on serines, and that cell extracts contain a relatively high PKC activity that can phosphorylate the channel in vitro (34). That PKC may be responsible for the resting phosphorylation of *n* channels in vivo is consistent with evidence that the K^+ current in Jurkat cells increases gradually during whole-cell recordings made with anti-PKC antibody or alkaline phosphatase in the recording pipette (51). The discrepancy between phorbol ester effects on Jurkat cells and human peripheral blood T cells remains unexplained. Nevertheless, these results raise the possibility that antigenic stimulation may inhibit *n* channels by generating diacylglycerol (DG) and activating PKC; thus, two downstream messengers of the antigen receptor, Ca^{2+} and DG, may act in concert to limit the number of open K(V) channels during lymphocyte activation.

EFFECTS OF cAMP The inhibitory effects of cAMP on T cell activation (37) have prompted several groups to investigate its actions on K^+ channels. A full spectrum of effects has been reported, ranging from channel inhibition, to no effect, to activation. Choquet et al (45) found that intracellular application of cAMP almost completely eliminated the type *n* K^+ current in murine B cell blasts and pre-B cell lines, and similar findings were later reported in Jurkat T cells treated with 8-Br-cAMP or the cAMP agonist PGE_2 (51). The effect in Jurkat cells was blocked by inhibitors of protein kinase A (PKA), suggesting a phosphorylation-dependent mechanism (51). As in the case of phorbol esters, the effects of cAMP in Jurkat cells do not extend in a consistent way to *n* channels in human peripheral T cells. Several studies found no effect of cAMP applied through the recording pipette or of bath-applied permeant cAMP analogs (43, 44, 52, but see 53), even at 37°C (40). Pahapill & Schlichter (44) addressed this discrepancy in a study of K^+ channels in cell-attached patches. Several agents that elevate cAMP levels induced a slow rise in K^+ channel activity at the resting potential. It is unknown whether these channels are modified *n* channels or are previously "silent" channels activated by cAMP. It should be noted that in another study PGE_2 failed to activate K^+ channels in cell-attached patches (40). The question remains why cAMP appears to inhibit K^+ channels in some cases and to activate them in others. Recent biochemical studies suggest that the actions of cAMP on the type *n* channel may be complex. In vitro PKA phosphorylates not only the Jurkat *n* channel, but also a 40-kDa protein with which it coprecipitates (34). This protein is similar in size to the recently characterized β subunit believed to accelerate the inactivation of Kv1 channels (50), and by analogy it may have a similar action on *n* channels in lymphocytes. Thus, a more complete biochemical characterization of native *n* channels and their associated subunits in different lymphocyte subtypes may shed light on the diversity of cAMP effects that have been described.

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EFFECTS OF TEMPERATURE Several properties of type *n* channels are profoundly temperature-dependent, implying that caution must be exercised when extrapolating from results obtained at room temperature to predict K^+-channel behavior under physiological conditions (40, 41). Increased temperature affects the overall activity of *n* channels in several ways: 1) It increases the single-channel conductance; 2) it accelerates activation, inactivation, and deactivation kinetics; 3) it shifts the voltage dependence of inactivation and activation in opposite directions, thereby increasing by two- to threefold the number of channels active at the resting potential; and 4) it speeds the recovery from inactivation. The net effect of these changes is to increase significantly the activity of K^+ channels at 37°C relative to room temperature (40, 41). This information will be essential in developing a realistic quantitative model of K^+-channel activity in lymphocytes under physiological conditions. Such modeling may provide the ultimate test of hypotheses regarding the functional roles of K^+ channels and their modulation in the intact lymphocyte.

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Ca^{2+}-Activated K^+ Channels

The existence of Ca^{2+}-activated K^+ [K(Ca)] channels in lymphocytes was first inferred from observations that Ca^{2+} ionophores increased the membrane K^+ permeability (measured with radioactive isotopes) and evoked membrane hyperpolarization (30, 54–56; for review, see 1, 57). Subsequent patch-clamp studies identified K(Ca) channels in rat thymocytes (58, 59), human and mouse B cells (58, 60, 61), and human T cells (62, 63). The current consensus is that at least two types of K(Ca) channels, readily distinguished by their different conductances and pharmacological profiles, are expressed in a lineage-specific pattern in lymphocytes (Table 1). A small-conductance (2–8 pS) "mini" channel appears to be absent from human T cells (63, but see 64), is rare in B cells (60), but is quite abundant in Jurkat cells (several hundred/cell) (62). This channel is effectively blocked by apamin but is insensitive to CTX (62). A larger, 10-35 pS "midi" channel is relatively more abundant in B cells (60), thymocytes (58), and peripheral blood T cells (63) and is rare in Jurkat (62). In contrast to the mini-K(Ca) channel, this larger channel is insensitive to apamin but is half-blocked by 3 nM CTX (60, 62, 63). Neither channel exhibits significant voltage dependence, unlike the large-conductance "maxi"-K(Ca) channel found in other cell types (65); instead, each is highly sensitive to intracellular Ca^{2+}. The calcium dependence of both channels has been measured in whole-cell recordings by intracellular perfusion with buffered Ca^{2+} solutions and by simultaneous patch-clamp and $[Ca^{2+}]_i$ measurements. Both channels are activated by $[Ca^{2+}]_i > 200$–300 nM, and activation by Ca^{2+} is cooperative, with a Hill coefficient of ≈ 4 (62, 63), suggesting that multiple Ca^{2+}-binding sites are involved in channel opening. The genes for these channels have not yet been cloned.

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K^+ Channel Expression During Development and Activation

T LYMPHOCYTES The surface density of K^+ channels in the plasma membrane of T lymphocytes is regulated in intriguing ways during development and during activation of mature cells by mitogens (for reviews, see 8, 29). Developmental regulation of K(V) channels is most pronounced in murine lymphocytes. Immature thymocytes ($CD4^-$ $CD8^-$ or $CD4^+$ $CD8^+$) express hundreds of type *n* channels per cell. As the developmental lineage diverges, *n* channels are either downregulated ~10-fold in $CD4^+$ $CD8^-$ cells (helper phenotype) or supplanted with a mixture of ≈20–200 *n'* and *l* channels in most $CD4^-$ $CD8^+$ cells (cytotoxic/suppressor subset) (13). Mitogenic activation causes both classes of mature murine T cells to revert to the immature pattern of channel expression, i.e. hundreds of *n* channels per cell (66, 67). In contrast, resting human T cells express *n* channels at a level similar to that of immature or activated T cells of mice, and activation increases channel abundance only by a factor of ≈2–3 (12, 63, 68). However, the expression of K(Ca) channels in human T cells is profoundly upregulated by mitogens, increasing from ≈20 per resting cell to >500 channels per cell in T cell blasts treated with phytohemagglutinin (PHA) (63). Thus, the relative contribution of K(Ca) channels to maintaining the membrane potential may be greatly enhanced in activated cells and perhaps in memory cells. Type *l* K(V) channels have been found only in $CD4^-CD8^+$ thymocytes and peripheral T cells from mice (at low levels) and at high levels in $CD4^-CD8^-$ T cells from a variety of autoimmune mice such as *lpr, gld,* NOD, and EAE (for review, see 8). Type *l* channels have not been detected in human T cells but are present in a human Burkitt's lymphoma cell line, Loucks (69).

Recent evidence suggests that the expression of K(V) channels during T cell activation is regulated through a posttranscriptional mechanism. Following mitogenic stimulation, Kv1.3 mRNA levels remain constant or decrease in murine and human T cells as the number of channels increases (18, 20). A number of posttranscriptional mechanisms could explain the increase in K^+-channel density, including an increased rate of translation, stimulated insertion of presynthesized channels, or unmasking of previously "silent" channels. Transcriptional control mechanisms cannot be ruled out, however, as several Kv1.3 transcripts have been detected [≈9.9, 4.4, and ≈3 kb (18, 20)], and the expression of the smaller two transcripts increases with time in activated Jurkat cells (18). The relative translational efficiency of the different transcripts is not known.

B LYMPHOCYTES Independent regulation of K(V) and K(Ca) channels via different activation pathways has been demonstrated in B cells (61). Stimulation through the antigen receptor increases expression of both channels, while lipopolysaccharide (LPS) affects only K(V) channels. In each case, the surface density of the channels increases roughly 5–10-fold to hundreds per cell. Inhibitors of RNA synthesis block the induction of K(Ca) channels by antigen,

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indicating a requirement for gene transcription. The functional implications of differential regulation are unknown, but the induction of K(Ca) channels appears to be correlated with an inability to differentiate (61). LPS elevates the levels of K(V) channels and triggers differentiation into plasma cells, while anti-μ induces K(Ca) channels in LPS-pretreated cells and inhibits their ability to differentiate. Identification of a specific blocker for midi-K(Ca) channels in B cells would offer a means of testing this hypothesis.

K(V) Channels as a Pharmacological Target

A prospective therapeutic intervention in immune disease is based on pharmacological inhibition of type *n* K^+ channels. These channels are blocked by a remarkable variety of agents, including classical K^+ channel blockers [TEA and 4-aminopyridine (4-AP)], Ca^{2+}-channel antagonists (verapamil and dihydropyridines), K(Ca) channel blockers (quinine and cetiedil), calmodulin antagonists (trifluoperazine and chlorpromazine), and the steroid hormone progesterone (for reviews, see 2, 7, 8). Many of these compounds also inhibit lymphocyte activation (below); however, their rather low affinity ($K_i = 10^{-5}$ to 10^{-2} M) and specificity undermine their usefulness either as research probes for K(V) channel function or as therapeutic tools. The more recent identification of peptide toxins from scorpion venoms that block K(V) and K(Ca) channels with high affinity and specificity offers great promise. CTX blocks type *n* K(V) as well as K(Ca) channels with a K_i of $\approx 10^{-9}$ M (60, 70, 71). Noxius toxin (NTX) (64, 70), margatoxin (MTX) (72), and kaliotoxin (KTX) (61) are even more potent and are also selective for K(V) over K(Ca) channels. It is encouraging to note that low, therapeutic concentrations of calcium antagonists that also block *n* channels (verapamil, nifedipine, nimodipine, and diltiazem) significantly enhance the immunosuppressive effects of cyclosporin A (CsA) in vitro (73). Thus, K^+-channel blockers may synergize with low doses of CsA in vivo to increase the survival of organ grafts while minimizing the nephrotoxicity and hypertension associated with conventional CsA therapy. To this end, several pharmaceutical companies are currently attempting to develop K(V) blocking agents of high affinity and specificity using both toxins and organic compounds. The success of this approach may be aided by the restricted tissue distribution of Kv1.3 mRNA, which is expressed abundantly in thymus and spleen, at a low level in fibroblasts, brain, and kidney, and not detectably in liver or heart (16, 19).

Functional Roles for K^+ Channels in Lymphocytes

CONTROL OF MEMBRANE POTENTIAL The membrane potential (V_m) exerts powerful effects on lymphocyte activation (see below). Based on measurements of radiolabeled and fluorescent membrane potential probes, the resting potential of quiescent T cells from humans and mice is −50 to −70 mV (56, 64, 74, 75; for review, see 1). In human T cells, the resting potential results

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primarily from a K^+-diffusion potential contributed by *n*-type K^+ channels. It has been estimated that on average only several *n* channels are open in resting T cells, and that this can account for the resting K^+ efflux (11, 41). This conclusion agrees well with the observation that the resting potential of T cells slowly fluctuates by ≈8 mV, presumably due to the opening and closing of single K^+ channels (76). The extremely high input resistance of resting T cells makes it possible for such a small number of open channels to determine the membrane potential and creates a substantial reserve of dormant K^+ channels to "protect" against depolarization. High-affinity blockers provide definitive pharmacological evidence for the role of *n* channels in setting the resting potential. CTX, margatoxin, and NTX depolarize resting human T cells (30, 64), and although CTX also blocks K(Ca) channels, its effect on V_m is no greater than that of the more selective K(V) blockers (64).

In murine T cells, the contribution of K(V) channels to establishing the resting potential correlates well with their level of expression (75). Type *n* channels play a dominant role in immature thymocytes or activated peripheral T cells, both of which express hundreds of channels per cell. In contrast, V_m in mature resting T cells (with ≈10 *n* channels/cell) is determined largely by the electrogenic activity of the Na^+/K^+ ATPase. Blocking the pump with ouabain, low temperature, or removal of K_o^+ depolarizes these cells, whereas moderate changes in the transmembrane K^+ gradient have little effect (75). The Na^+/K^+ pump's contribution to V_m may explain why CTX does not depolarize rat thymocytes (55), cells that express low numbers of type *n* channels (M Cahalan, unpublished observation).

K(Ca) channels do not appear to contribute significantly to V_m in resting lymphocytes but exert a powerful influence when $[Ca^{2+}]_i$ is elevated. $[Ca^{2+}]_i$ in resting lymphocytes is 50–100 nM (1), well below the measured threshold for activating K(Ca) channels (200–300 nM) (58, 62, 63). K(Ca) channels are silent in cell-attached recordings from intact resting lymphocytes except during a brief period immediately after establishing the membrane-pipette seal, when a $[Ca^{2+}]_i$ spike often occurs (58, 59). The rise in $[Ca^{2+}]_i$ elicited by mitogens or Ca^{2+} ionophores is sufficient to activate K(Ca) channels in both whole-cell and cell-attached configurations (58, 59, 62, 63). Thus activated, K(Ca) channels cause a membrane hyperpolarization that is prevented or reversed by CTX (55, 59, 77) or quinine (56), both of which block K(Ca) channels in patch-clamp studies (60, 63).

REGULATION OF CELL VOLUME When exposed to hypoosmotic solutions, T cells swell rapidly, then shrink over several minutes back to approximately their normal size. This regulatory volume decrease (RVD) response is mediated by the stimulated efflux of K^+ and Cl^-, which drives osmotically obligated water from the cell (1). A model for RVD in T cells has been proposed in which cell swelling activates Cl^- channels (78), eliciting an efflux of Cl^- that depolarizes

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the membrane and thereby opens type *n* K(V) channels (31, 32). Several types of evidence support a role for type *n* channels in RVD. First, the density of *n* channels in several types of lymphocytes correlates with the ability to volume regulate (75, 79). Second, a series of pharmacological reagents block *n* channels and inhibit RVD with the same relative order of potency (reviewed in 31, 32). Finally, increasing the expression of type *n* channels by mitogenic stimulation of murine T cells (79) or by transfection of Kv1.3 into the K^+-channel and RVD-deficient CTLL-2 cell line (21) confers the ability to volume regulate. Significantly, *l* channels expressed by CTLL-2 cells after transfection with Kv3.1 did not fulfill this role (21). These results present compelling evidence for the roles of swelling-activated Cl^- channels and type *n* K^+ channels in RVD, as Cl^- channels can only depolarize the cell to a maximum of ≈ -35 mV (the estimated equilibrium potential for Cl^-), above the activation threshold for *n* channels (-60 mV) but well below that of type *l* channels (-10 mV). K(Ca) channels normally do not appear to be involved in RVD but can assume this function in murine T cells treated with ionomycin (75).

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ACTIVATION OF LYMPHOCYTES Two types of circumstantial evidence support a role for K^+ channels in the activation of lymphocytes. First, as described above, T and B cell mitogens stimulate an increase in the density of K(V) and K(Ca) channels, and the density of K^+ channels in thymocyte subsets is positively correlated with proliferative activity. Second, pharmacological studies suggest a requirement for functional K^+ channels in the activation of T and B cells. A variety of chemically distinct K(V) channel blockers, including TEA, 4-AP, quinine, verapamil, diltiazem, cetiedil, trifluoperazine, and chlorpromazine, inhibit activation, gene expression, killing by cytotoxic T cells and NK cells, lymphokine secretion, and proliferation (4, 80–82). The discovery that K^+-channel blockers can inhibit proliferation has since been extended to a variety of cell types outside the immune system (reviewed in 83, 84).

The interpretation of results obtained with K^+-channel blockers is complicated by the fact that some of these compounds have effects on proteins other than K(V) channels. For example, TEA and quinine also block K(Ca) channels, and trifluoperazine is a potent inhibitor of calmodulin. The identification of high affinity, specific K^+-channel blockers from scorpion venoms has allowed more definitive studies of K^+-channel functions in intact cells. Deutsch and colleagues first demonstrated that nanomolar concentrations of CTX effectively inhibit PHA- or antigen-driven T cell activation in vitro (71). The inhibition appears to result from suppression of IL-2 transcription, as the level of IL-2 mRNA and secreted IL-2 are both reduced in CTX-treated cultures (85), and normal proliferation can be restored by supplying exogenous IL-2 (71). Because CTX blocks K(V) and K(Ca) channels with similar potency, it cannot be used to distinguish their functional roles. Lin et al (86) addressed this

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point using the selective K(V)-channel blockers, margatoxin (MTX) and noxiustoxin (NTX); like CTX, both peptides block mitogen-induced proliferation, the mixed lymphocyte response, and the secretion of IL-2 and γ-IFN. Moreover, MTX inhibits IL-2 secretion at lower concentrations than does CTX, consistent with its higher affinity for the type *n* K^+ channel. These results provide the strongest available evidence for a role of K(V) channels in mitogenesis. However, even though CTX and NTX block activation with equal efficacy (86), it should be noted that the effects of these peptides do not rule out a role for K(Ca) channels downstream from events involving K(V) channels. At present the absence of a selective blocker for K(Ca) channels precludes a test of this possibility.

Considerable evidence suggests that functional K^+ channels are required during T cell activation to maintain a membrane potential that promotes Ca^{2+} influx. A negative membrane potential enhances Ca^{2+} entry by optimizing the electrochemical driving force for Ca^{2+} movement through Ca^{2+} channels; in addition, indirect effects of V_m on the activity of the Ca^{2+} channels themselves have also been suggested (87–89). Several types of evidence support a V_m-mediated link between K^+ channels, Ca^{2+} influx, and cell activation. High affinity K^+-channel antagonists like CTX, NTX, and MTX depolarize human T cells (30, 64) and inhibit T cell activation induced by Ca^{2+}-dependent pathways (anti-CD2 or anti-CD3) but not Ca^{2+}-independent ones (anti-CD28+ PMA, or IL-2) (86). The immunosuppressive effects of CTX in vitro can be mimicked by an equivalent depolarization invoked directly by elevated $[K^+]_o$ (85). Furthermore, depolarization inhibits the $[Ca^{2+}]_i$ rise induced by T cell mitogens (88, 90–93), whereas hyperpolarization by the potassium ionophore valinomycin reverses the effect of K^+-channel blockade on $[Ca^{2+}]_i$ (87).

While these studies suggest that K(V) channels are needed to enhance Ca^{2+} entry, several unresolved discrepancies remain. First, Gelfand & Or (77) found that CTX had no effect on either the mitogen-induced $[Ca^{2+}]_i$ rise or activation of human lymphocytes, even though it effectively blocked RVD. Second, even doses of CTX more than tenfold greater than the K_i for K^+-channel blockade inhibit activation by only 50–60% (71, 85, 86). This may be due to the fact that CTX evokes a maximal depolarization of only 20–25 mV (30, 64), implying that CTX-insensitive K^+ or Cl^- channels also contribute to the resting potential, particularly when *n* channels are blocked. Contributions of CTX-insensitive mechanisms to membrane potential might also explain the lack of CTX effects observed by Gelfand & Or (77). Finally, in the study by Lin et al (86), although CTX did not affect the long-lasting plateau level of $[Ca^{2+}]_i$, it inhibited activation significantly even when added hours after the mitogen, i.e. well into the plateau phase. In this regard, it should be noted that $[Ca^{2+}]_i$ oscillations may be important in controlling lymphocyte activation (94), and

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these would not be detected in the population $[Ca^{2+}]_i$ measurements discussed above. Blockade of K(V) and K(Ca) channels with CTX and apamin effectively inhibits PHA-triggered $[Ca^{2+}]_i$ oscillations in Jurkat cells, but it has only a small effect (≈25% decrease) on the average $[Ca^{2+}]_i$ of the cell population (62). Thus, further studies of the effects of channel antagonists on $[Ca^{2+}]_i$ at the single-cell level may better define the role of K(V) channels in Ca^{2+} signaling and lymphocyte activation.

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CALCIUM CHANNELS IN LYMPHOCYTES

A rise in $[Ca^{2+}]_i$ is an essential triggering signal for many of the events associated with lymphocyte activation. These events include changes in motility and cell shape occurring soon after T cells contact antigen-presenting cells (95, 96), transcription of genes for lymphokines such as IL-2 (97–99), cell killing by cytotoxic T cells (100, 101), apoptosis of self-reactive T cells during development in the thymus (102, 103), and induction of tolerance to self-antigens in mature peripheral T cells (104, 105). In the past several years, substantial progress has been made in understanding many of the key molecular events upstream and downstream of the Ca^{2+} signal. Binding of antigen (or mitogens) to the TCR activates multiple tyrosine kinases and leads to the phosphorylation and activation of phospholipase Cγ1 (for reviews, see 39, 106). Once activated, the enzyme cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the second messengers IP_3 and diacylglycerol (DG). IP_3 evokes a rise in $[Ca^{2+}]_i$, which in combination with DG activates PKC. The elevation of $[Ca^{2+}]_i$ appears to be necessary and, together with PKC, sufficient to drive many events in T cell activation (for reviews, see 36, 97). Ca^{2+} exerts much of its influence through the activation of transcription factors that control the production of lymphokines such as IL-2 (99, 107). Additional signaling molecules such as ras operate within parallel pathways to play critical roles in T cell activation, but these are beyond the scope of this review (107, 108).

Because of its central role in lymphocyte activation, an intense effort has been focused on defining the underlying basis of the Ca^{2+} signal. The first phase of the $[Ca^{2+}]_i$ rise is generally understood to be mediated by IP_3 binding to its receptor in the endoplasmic reticulum (ER) membrane and opening a Ca^{2+} channel that releases stored Ca^{2+} into the cytosol (109). The second phase, considerably less well understood, consists of a prolonged influx of Ca^{2+} across the plasma membrane. Because of the finite capacity of the ER Ca^{2+} store, intracellular release generates a transient signal that is neither necessary nor sufficient to drive activation (110, 111). Ca^{2+} influx and elevation of $[Ca^{2+}]_i$ must be sustained for >30 min to commit T cells to become activated and express the IL-2 gene (94, 107, 111). Considerable effort has been spent over the past few years to identify the Ca^{2+} channels that carry out this signaling function and to understand the mechanisms that regulate their activity.

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Identification of Mitogen-Regulated Calcium Channels

A great variety of channels have been proposed to mediate mitogenic Ca^{2+} influx in lymphocytes, an indication of both the intense interest in elucidating this pathway and the technical difficulties inherent in doing so. Below, we consider many of the proposed mechanisms in the context of the characteristics of the Ca^{2+} influx pathway, as inferred from $[Ca^{2+}]_i$ measurements in intact cells (90, 93, 109, 112, 113). Based on these studies, any potential candidate for the mitogen-activated Ca^{2+} channel should exhibit the following properties: 1) activation by mitogens or downstream messengers (e.g. IP_3); 2) significant Ca^{2+} permeability and an ability to carry Mn^{2+}; 3) activity that is temporally correlated with increases in $[Ca^{2+}]_i$; 4) decreased Ca^{2+} conductance upon depolarization; 5) feedback inhibition by intracellular Ca^{2+}; and 6) inhibition by Ni^{2+} and imidazole antimycotics such as SKF 96365. So far, these six criteria have been met only for depletion-activated Ca^{2+} channels.

DEPLETION-ACTIVATED CALCIUM CHANNELS These channels were first described by Lewis & Cahalan (93) in Jurkat T cells using whole-cell recording and simultaneous $[Ca^{2+}]_i$ imaging with fura-2. A small, highly selective Ca^{2+} current appeared spontaneously during intracellular dialysis with solutions containing EGTA. The current displayed many of the properties expected for mitogenic Ca^{2+} channels, including high permeability to Ca^{2+}, a tight temporal link to changes in $[Ca^{2+}]_i$, inhibition by depolarization, evidence of Ca^{2+}-dependent feedback inhibition, and blockade by Ni^{2+} and Cd^{2+}. In perforated-patch recordings, the current did not develop spontaneously but could be activated by PHA in an oscillatory manner. A calculation based on the lymphocyte volume demonstrated that the observed 1- to 10-pA Ca^{2+} current was sufficiently large to account for the observed rate at which $[Ca^{2+}]_i$ rises during oscillations in PHA-stimulated cells (≈100 nM/sec maximum) (93), even allowing for substantial binding of Ca^{2+} by cytoplasmic buffers (114). The mode of regulation for these channels was unclear at the time; although activation by PHA could be explained through the action of IP_3, activation by intracellular dialysis alone was unlikely to involve the same messenger. The ability of IP_3 to activate a similar Ca^{2+} current in Jurkat cells was later shown directly by flash photolysis of intracellular caged IP_3 (115).

The key to understanding this Ca^{2+} channel came from the capacitative Ca^{2+} entry hypothesis originally proposed by Putney to explain receptor-mediated Ca^{2+} influx in exocrine cells (116). According to this hypothesis, depletion of the ER Ca^{2+} store generates a signal that activates Ca^{2+} influx across the plasma membrane. Supporting evidence has accumulated rapidly since the isolation of compounds that block Ca^{2+}-ATPases in the ER membrane and thereby unmask a passive Ca^{2+} leak that empties the stores without generating IP_3. Store depletion by several of these compounds, including thapsigargin (TG), tert-butylhydroquinone (tBHQ), and cyclopiazonic acid (CPA), activates

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Ca^{2+} influx in rat thymocytes and human T cells (113, 117–119), as does store depletion induced simply by prolonged incubation of cells in Ca^{2+}-free media (113, 120). Overlap between the depletion-activated and mitogen-activated Ca^{2+} entry mechanisms was first indicated by observations that TG depletes the same Ca^{2+} store that is mobilized by TCR ligation (112, 113, 117, 119), and that stimulation of the TCR fails to evoke an additional $[Ca^{2+}]_i$ increase in cells pretreated with a maximal dose of TG (113, 119). In view of these results, Zweifach & Lewis (121) used perforated-patch recording to test whether the mitogen-regulated Ca^{2+} current is activated by store depletion, by comparing the Ca^{2+} currents activated by TG and PHA in Jurkat T cells. The two currents were identical in every property that was tested, including divalent ion selectivity, voltage-independence of gating, sensitivity to blockade by Ni^{2+}, and single-channel conductance. The single-channel conductance was estimated from noise analysis to be ≈24 fS in 110 mM Ca^{2+}_o; this extremely small value and the channel's high Ca^{2+} selectivity readily distinguish it from IP_3-gated Ca^{2+} permeable channels previously described in T cells (see below; 122, 123). The simplest conclusion consistent with these results is that mitogen-activated Ca^{2+} channels in T cells are regulated by the depletion of Ca^{2+} stores, and that under physiological conditions of TCR stimulation, IP_3 activates Ca^{2+} channels through store depletion rather than by a direct action on the Ca^{2+} channels themselves. This conclusion was later confirmed by Gardner and colleagues (124) in a comparison of whole-cell Ca^{2+} currents in Jurkat cells treated with TG, tBHQ, CPA, and intracellular IP_3; in particular, the TG-induced current was not increased further by IP_3-mobilizing agents and vice versa, consistent with previous evidence that the two stimulus pathways converge upon the same channel (113, 119). Choquet and coworkers have recently described an apparently identical depletion-activated Ca^{2+} current in human peripheral blood T cells (125). Thus, present evidence favors the idea that stimulation of the TCR triggers an influx of Ca^{2+} through low conductance, voltage-independent Ca^{2+} channels that are controlled indirectly by IP_3-driven depletion of the intracellular Ca^{2+} stores.

Although additional Ca^{2+} entry pathways activated by the TCR may exist, most evidence argues otherwise. First, stimulation through the TCR fails to elevate $[Ca^{2+}]_i$ or the Ca^{2+} current when added after an optimal dose of TG (113, 119, 124; but see 126). Second, antigen-unresponsive T cells from patients with a primary immunodeficiency lack the sustained mitogen-driven increase in $[Ca^{2+}]_i$ in conjunction with an absence of depletion-activated Ca^{2+} current (125). Taken together, these findings suggest that depletion-activated Ca^{2+} channels play the major and perhaps the sole role in mediating the influx of Ca^{2+} essential for T cell activation.

IP$_3$-GATED CHANNELS Kuno et al (122) were the first to report PHA-activated single-channel Ca^{2+} and Ba^{2+} currents in cell-attached patches from human

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T cells. Similar currents were observed in excised patches exposed to IP_3 on the intracellular face, leading to the conclusion that the channels were directly activated by this messenger (123). These channels fulfill several of the criteria for being mitogenic Ca^{2+} channels: They are activated by cross-linking of the TCR as well as by IP_3; they conduct current in the presence of isotonic Ca^{2+} or Ba^{2+}; they exhibit voltage-independent gating, and the current amplitude decreases with depolarization; and they are inhibited by micromolar $[Ca^{2+}]_i$ and by Cd^{2+} (122, 123, 128). Moreover, Khan et al (129) have detected IP_3R-like immunoreactivity on the surface of human T cells by means of an antiserum generated against the cerebellar microsomal IP_3 receptor. Concanavalin A caps the immunoreactivity as well as Ca^{2+} influx sites as inferred from elevated $[Ca^{2+}]_i$ near the cap (129). However, several necessary criteria supporting a role of the IP_3 receptor in the influx of Ca^{2+} remain untested. First, the single-channel currents appeared to have a low selectivity for Ca^{2+} over monovalent ions (123), raising the question as to whether they have the capacity to conduct significant amounts of Ca^{2+} under physiological conditions. A correlation of their activity with the increase in $[Ca^{2+}]_i$ has not been demonstrated. Second, activity of IP_3-gated channels has not been observed in whole-cell recordings, suggesting that the density of these channels is quite low. Even in the relatively noninvasive perforated-patch mode, electrical noise expected from the opening and closing of 8-pS IP_3-gated channels was undetectable in PHA-treated cells (121). Although IP_3 activates a Ca^{2+} current in whole-cell recordings from Jurkat cells (115), this appears to be identical to the depletion-activated Ca^{2+} current (121, 124). Third, the correlation of IP_3R-like immunoreactivity and gradients of $[Ca^{2+}]_i$ in capped cells may be misleading. Apparent gradients often result from the uptake of fura-2 by organelles, which can be significant in human T cells (130) and could underlie the spatial non-uniformity of the total fura-2 concentration that was observed (129). Furthermore, con A caps a large number of membrane glycoproteins in T cells, including perhaps the depletion-activated Ca^{2+} channel. For these reasons, it appears unlikely that plasma-membrane IP_3 receptors mediate significant Ca^{2+} entry, and their physiological function in T cells remains unclear (124).

OTHER POSSIBLE CALCIUM CHANNELS IN LYMPHOCYTES Two lines of evidence suggest that voltage-dependent Ca^{2+} channels are not expressed at a significant level in T cells. First, depolarization of resting Jurkat cells or human T cells with high $[K^+]$ (87, 88, 91–93, 131) or by current injection (132) fails to raise $[Ca^{2+}]_i$ significantly; and second, several groups have failed to observe voltage-gated Ca^{2+} currents under whole-cell recording conditions optimal for their detection in excitable cells (122, 133, 134). However, there are two reports of voltage-gated Ca^{2+} currents in whole-cell recordings from T cell lines. In one case, a large, slowly inactivating inward current was activated at potentials above −60 mV in a subset of Jurkat 77 T cells (135), while in another a small,

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rapidly inactivating inward current was reported in Jurkat, Molt-4, and HSB T cell lines (136). The second current was active transiently (duration < 10 msec) at potentials above −20 mV, while it was inactivated at potentials more positive than −90 mV; these properties appear to be inconsistent with a role in mediating a sustained rise in $[Ca^{2+}]_i$ at physiological potentials of −50 to −80 mV. There is no direct evidence to show that either current is carried by Ca^{2+}, and although the size of both of these currents appears to be enhanced by stimulation of TCR, significant activity of the channels at physiological membrane potentials has not been demonstrated. Several experiments could clarify the existence and role of these channels in T cells: if they are to mediate mitogenic Ca^{2+} influx, then Ca^{2+}_0 removal should eliminate the current, depolarization should increase $[Ca^{2+}]_i$, and holding the cell at potentials below the channel's threshold for activation should prevent the $[Ca^{2+}]_i$ increase triggered by mitogens.

A Ca^{2+} entry pathway independent of the phosphatidylinositol cascade has been proposed based on the ability of mitogens to evoke Ca^{2+} influx in HPB-ALL cells without measurable production of inositol phosphates or release of intracellular Ca^{2+} (137). In lipid vesicles reconstituted from HPB-ALL plasma membrane, anti-CD3 mAb induced Ca^{2+} uptake to a greater extent than nonmitogenic control antibodies to CD2 or Thy 1.2; this was specifically inhibited by several conditions that reduce influx in intact HPB-ALL cells. It is not known whether this pathway can account for Ca^{2+} influx in intact HPB-ALL cells, nor whether it also exists in nontransformed cells. Another Ca^{2+} entry mechanism distinct from the TCR-triggered pathway has been reported in T cells from patients with multiple sclerosis. γ-interferon activates Ca^{2+} entry in these cells apparently through activation of protein kinase C (138). Patch-clamp studies may help to clarify the nature of these additional routes for Ca^{2+} entry.

CD20, a B cell plasma membrane phosphoprotein thought to play a role in B cell activation and cell-cycle progression, has been proposed to be a Ca^{2+} channel (139). The evidence for this is indirect, based largely on the appearance of an inwardly rectifying Ca^{2+} current and an increased resting Ca^{2+} permeability in cells transfected with CD20, and an acute increase in whole-cell conductance induced by an anti-CD20 mAb. Several key questions about the nature and function of CD20 remain. First, the increase in conductance induced by cross-linking CD20 is nonselective and thus distinct from the resting CD20-associated conductance. Second, the authors do not exclude the possibility that CD20 is a channel modulator rather than a channel itself. In fact, other work has shown that mAb binding to CD20 initiates tyrosine kinase activity and oncogene expression (140, 141), actions suggestive of a biochemical modulator. Finally, the mechanisms that control CD20-linked Ca^{2+} entry under physiological conditions are unknown, although a link to depletion-activated Ca^{2+} channels has been proposed (139).

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Properties of Depletion-Activated Calcium Channels

The evidence reviewed above strongly supports the conclusion that depletion-activated Ca^{2+} channels underlie mitogenic Ca^{2+} influx in T cells. Although their properties differ from those of voltage-gated Ca^{2+} channels in electrically excitable cells, T cell Ca^{2+} channels closely resemble Ca^{2+} release-activated Ca^{2+} (CRAC) channels originally described in rat mast cells by Hoth & Penner (142, 143). Patch-clamp studies indicate that these channels are present in a wide variety of cell types, including RBL cells, fibroblasts, thyrocytes, hepatocytes, HL-60 cells, and *Xenopus* oocytes (144–146); indirect evidence based on Ca^{2+}-sensitive dyes suggests an even wider distribution (116). A complete understanding of the capacitative Ca^{2+} entry pathway in lymphocytes and other cells will require cloning of the CRAC channel and other elements involved in its regulation. The unique nature of the channel and its mode of activation, a frustrating lack of high-affinity ligands, and an extremely widespread tissue distribution make its cloning a challenging goal. The defining properties of CRAC channels are reviewed below with particular emphasis on T cells and mast cells, in which they have been studied most extensively. This channel "fingerprint" may prove to be useful for the identification of these channels in other immune cells.

ION SELECTIVITY CRAC channels are extremely selective for Ca^{2+} over monovalent cations, having a relative Ca^{2+} permeability comparable to that of voltage-gated Ca^{2+} channels (121, 124, 143). The divalent conductance sequence for the channel is $Ca^{2+} > Ba^{2+} \simeq Sr^{2+} >> Mn^{2+}$, with Ba^{2+} and Sr^{2+} carrying current about 50% as well as Ca^{2+}. This sequence is unusual, as most voltage-gated Ca^{2+} channels conduct Ba^{2+} and Sr^{2+} better than Ca^{2+} (147).

PHARMACOLOGY No specific, high-affinity blockers of I_{CRAC} (the current through CRAC channels) have been identified. A variety of organic antagonists of Ca(V) channels do not affect the current (124, 148), but imidazole antimycotics such as econazole and SKF 96365 inhibit capacitative Ca^{2+} influx (149, 150) and I_{CRAC} (148, 150). These compounds block with relatively low affinity (K_i values of 0.6 μM for econazole and 4–12 μM for SKF 96365) and in mast cells block nonselective cation channels and chloride channels with similar potency (148). Divalent (Ni^{2+}, Co^{2+}, Cd^{2+}, Mn^{2+}) and trivalent (La^{3+}) ions also inhibit I_{CRAC} in the range of 0.1–5 mM (93, 121, 124, 143), but they block type *n* K(V) channels at similar concentrations (134, 135, 151) and therefore cannot be used to elucidate the function of CRAC channels in intact cells.

UNITARY CONDUCTANCE AND LEVEL OF EXPRESSION One hallmark of the CRAC channel is its extremely small single-channel conductance [10–24 fS in T cells (121) and $<$1 pS in mast cells (143)]. This conductance is $\approx$100-fold smaller than that of voltage-gated Ca^{2+} channels, far too small for the single-

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channel currents to be detected in membrane patches or even for whole-cell current noise to be visible by eye. In principle, the low transport rate predicted by the unitary conductance could be achieved by a single-ion transport mechanism like an ion exchanger. Na^+/Ca^{2+} exchange has in fact been proposed to underlie Ca^{2+} entry in T cells (152), but such a mechanism cannot explain observations that Ca^{2+} influx decreases with depolarization (93) and is insensitive to the removal of $Na^{2+}{}_o$ (114, 153). The shape of its noise spectrum suggests that I_{CRAC} is carried by channels rather than by a single-ion transport mechanism, and based on the size of the unitary and whole-cell currents, a typical Jurkat cell may express > 10,000 CRAC channels (121).

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ACTIVATION AND INACTIVATION The gating of depletion-activated Ca^{2+} channels is their least well-understood feature and perhaps the most important from a physiological viewpoint. CRAC channels are not opened directly by depolarization (93, 115, 121, 124, 142), IP_3 (121, 124, 142), or IP_4 (124, 142). Most attention has been focussed on the possibility of activation by a diffusible messenger released from intracellular Ca^{2+} stores, although activation by physical contact between proteins in the ER and plasma membranes or by stimulated insertion of new channels is also possible. One hypothesis is that Ca^{2+} entry in T cells is directly driven by Ca^{2+} released from intracellular stores (154–156), based in large part on the ability of high concentrations of intracellular Ca^{2+} buffers to abolish capacitative Ca^{2+} entry (154–156; but see 110). The strongest evidence against Ca^{2+}-dependent activation is that I_{CRAC} is induced in whole-cell recordings by intracellular dialysis with Ca^{2+} buffers that clamp $[Ca^{2+}]_i$ to <10 nM, well below the resting level (93, 124, 142). In addition, TG or IP_3 activates I_{CRAC} in advance of the $[Ca^{2+}]_i$ rise in buffer-loaded cells (93, 115, 121, 124, 142). Thus, it appears that CRAC channels are opened by a signal other than Ca^{2+} itself, although modulatory roles for Ca^{2+} should not be excluded (see below).

The ability of several imidazole inhibitors of cytochrome P450 to inhibit capacitative Ca^{2+} entry originally led to the suggestion that a P450 metabolite is the activating messenger (157). However, a subsequent study has shown that chemical modification of these compounds can greatly reduce the anti-P450 activity without altering the inhibition of Ca^{2+} influx, suggesting that the imidazoles may block CRAC channels directly (158). A more recent candidate for the CRAC channel messenger is CIF (Ca^{2+} Influx Factor), a low-mol-wt phosphate-containing molecule present in Jurkat cell extracts (159). CIF activity is released from the microsomal fraction into the cytosol following depletion of Ca^{2+} stores, and when applied to several nonlymphoid cell types elicits a small, fluctuating $[Ca^{2+}]_i$ rise without triggering intracellular Ca^{2+} release. These results are consistent with a role for CIF as the messenger that couples ER depletion to CRAC channel activation. Confirmation will require the demonstration that purified CIF activates I_{CRAC} when applied

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intracellularly, for example through a patch pipette. The molecular identity of CIF is not known, although present data argue against a wide variety of known messengers (159). Other mechanisms involving small G proteins (160, 161), phosphatases (144), tyrosine kinases (162, 162a, 162b), nitric oxide (163), and cGMP (163, 164) have also been proposed to evoke capacitative Ca^{2+} entry in various cells, but further experiments are needed to assign a specific action to these in lymphocytes.

Ca^{2+} entering the cell through CRAC channels feeds back to inhibit the channel's activity in several ways. Rapid inactivation of the channels occurs over tens of msec (143, 165) and is driven by local intracellular Ca^{2+} accumulation and binding to sites located probably on the channel itself (165). In addition, increased $[Ca^{2+}]_i$ slowly inhibits I_{CRAC} over tens of seconds (93, 115, 121, 166). Part of this slow inactivation process is due to uptake of Ca^{2+} by stores, as it is blocked by TG (166). A second type of slow Ca^{2+}-dependent inhibition appears to involve a protein phosphatase, as it is sensitive to okadaic acid, a phosphatase inhibitor (166; A Zweifach, R Lewis, unpublished observations). Slow activation and inactivation of I_{CRAC} is likely to play a major role in generating oscillations of $[Ca^{2+}]_i$ (93, 114, 130). In addition, negative feedback by Ca^{2+} may act as an essential brake that prevents inappropriate or excessive increases in $[Ca^{2+}]_i$. These regulatory mechanisms may suggest useful therapeutic strategies for controlling lymphocyte responsiveness in vivo.

Receptor-mediated inhibition of capacitative Ca^{2+} entry has been described in B cells. Cross-linking of Fcγ type II receptors (FcγRII) to membrane immunoglobulin (mIg) inhibits anti-μ-induced influx of Ca^{2+} without preventing its release (167, 168). Because this treatment does not affect TG-induced Ca^{2+} entry (168), it probably does not inactivate the CRAC channel or preempt its activation signal. It is possible that stimulation of FcγRII enhances pumping and reuptake of Ca^{2+} by the stores and thereby prevents the activation of I_{CRAC} by anti-μ (D Choquet, personal communication). Cross-linking of FcγRII to mIg in vivo may contribute to the inhibition of B cell activity during the late phases of the humoral immune response, when concentrations of circulating antibody are high (167).

Lymphocyte Signaling by Calcium Oscillations

Single-cell measurements of $[Ca^{2+}]_i$ with fluorescent dyes have revealed a remarkable degree of dynamic behavior that is undetectable in cell populations. A number of studies have shown that stimulation of the TCR by mitogens or antigen elicits pronounced $[Ca^{2+}]_i$ oscillations in T cells and related cell lines (93, 95, 114, 169). In many cells, including antigen-stimulated B cells (170), $[Ca^{2+}]_i$ oscillations appear to result from the repetitive release and reuptake of Ca^{2+} by intracellular stores (171, 172). T cell oscillations are unusual in that they absolutely require Ca^{2+} influx, which is believed to occur through CRAC channels (93, 114). In single Jurkat cells stimulated with PHA, I_{CRAC} oscillates

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in tandem with periodic changes in $[Ca^{2+}]_i$ (93). A recent study suggests how the properties of CRAC channels may explain this behavior (130). Moderate release of stored Ca^{2+} by low doses of Ca^{2+}-ATPase inhibitors or a Ca^{2+} ionophore effectively induces $[Ca^{2+}]_i$ oscillations in human T cells similar to those triggered by mitogens. Both Ca^{2+} influx and the content of intracellular stores fluctuate in oscillating cells, supporting an oscillation model based on cross-communication between Ca^{2+} stores and CRAC channels in the plasma membrane (130). According to this model, a small Ca^{2+} leak from the ER, e.g. induced by IP_3, causes partial depletion that activates a fraction of the cell's CRAC channels. The ensuing influx elevates $[Ca^{2+}]_i$, promoting store refilling and subsequent channel closure. Ca^{2+} pumps in the plasma membrane then reduce $[Ca^{2+}]_i$ once more, enhancing efflux from the ER and triggering the next cycle. An essential element of any oscillation model is the presence of strong positive and negative feedback. K(Ca) channels may contribute feedback by generating membrane potential oscillations that would enhance Ca^{2+} influx during the rising phase (through hyperpolarization) and would diminish Ca^{2+} influx during the falling phase [as K(Ca) channels close and the membrane depolarizes]. Membrane-potential oscillations of this kind have been seen to accompany $[Ca^{2+}]_i$ oscillations in PHA-stimulated Jurkat cells (173).

What is the physiological function of $[Ca^{2+}]_i$ oscillations in T cells? A recent study addressed this question using an IL-2 reporter gene to determine the calcium dependence of IL-2 gene expression in single murine T hybridoma cells (94). IL-2 transcriptional activity was monitored with a *lacZ* gene driven by the NF-AT (Nuclear Factor of Activated T cells) enhancer, and $[Ca^{2+}]_i$ was elevated to steady levels using high doses of TG or allowed to fluctuate by stimulating with anti-CD3. In the presence of PMA, transcription was very sensitive to $[Ca^{2+}]_i$; a threefold increase in steady state $[Ca^{2+}]_i$ activated transcription in half the cells. In response to stimulation through the TCR, cells expressing *lacZ* tended to have larger and more frequent $[Ca^{2+}]_i$ spikes than *lacZ*$^-$ cells. These results show at a single-cell level the dependence of gene expression on $[Ca^{2+}]_i$ and suggest that $[Ca^{2+}]_i$ oscillations may serve to enhance the efficiency of signaling from the TCR to the nucleus.

TOWARD A MOLECULAR MECHANISM OF ANTIGEN-TRIGGERED CALCIUM SIGNALING

Progress over the past several years in understanding ion channels, Ca^{2+} regulation, and gene transcription in T cells suggests a model that links antigen recognition at the cell surface to the expression of specific genes in the nucleus through changes in $[Ca^{2+}]_i$ and PKC activity (Figure 1). Ca^{2+}-dependent signaling in lymphocytes may be considered to occur in three phases. First, molecular events at the cell surface commence with the binding of the peptide-MHC complex to the antigen receptor and include the subsequent stimulation

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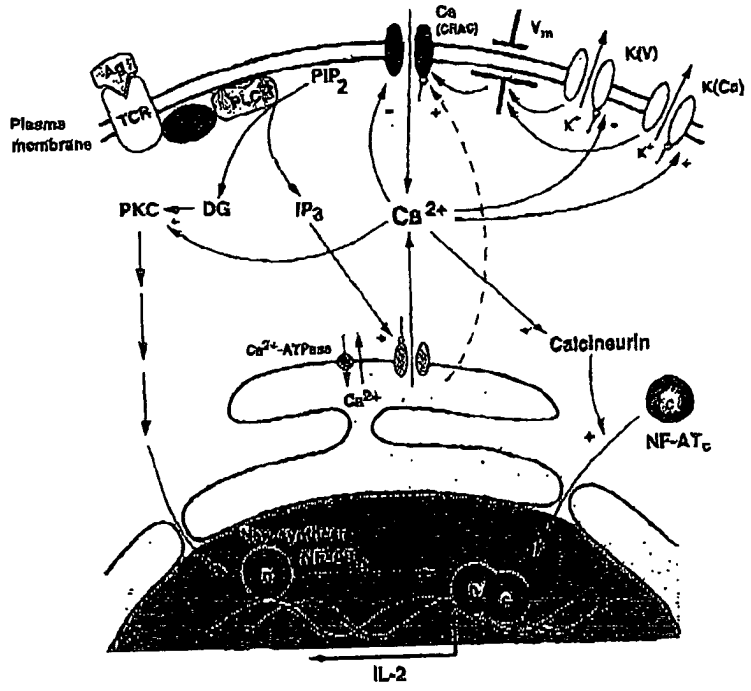

Figure 1 A cartoon of early events in T cell activation. Selected events from antigen binding to changes in ion channel activity to IL-2 gene expression are shown. Arrows labeled with + or − indicate stimulatory or inhibitory interactions, respectively.

of tyrosine kinases, phosphorylation and activation of PLCγ1, and hydrolysis of PIP_2 to produce IP_3 and diacylglycerol (reviewed in 106, 107). During the second phase, IP_3 carries the activation signal to the ER, where it releases stored Ca^{2+} through IP_3-activated channels. Depletion of stores triggers the opening of Ca^{2+} channels in the plasma membrane, leading to Ca^{2+} entry. Type *n* K(V) channels ensure the proper initiation of Ca^{2+} entry by maintaining the electrical gradient that helps drive Ca^{2+} into the cell. As $[Ca^{2+}]_i$ rises, K(Ca) channels open and may assume control of the membrane potential as K(V) channels undergo Ca^{2+}-dependent inactivation. Delayed communication between the ER and plasma-membrane CRAC channels, together with positive and negative feedback provided by K(Ca) channels and Ca^{2+}, may generate $[Ca^{2+}]_i$ oscillations under moderate stimulation conditions. The third phase of signal transduction connects these cytoplasmic events with gene transcription in the nucleus and probably occurs concomitantly with the first and second phases described above (98, 99, 107). Activated by diacylglycerol in concert with increased $[Ca^{2+}]_i$, PKC stimulates de novo synthesis of the nuclear-targeted component of NF-AT ($NF\text{-}AT_n$). In addition, the rise in $[Ca^{2+}]_i$ drives the

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Ca^{2+}/calmodulin-dependent activation of the phosphatase calcineurin. Dephosphorylation of one or more substrates by calcineurin in turn promotes the translocation of a preexisting cytosolic NF-AT_c subunit across the nuclear membrane, where it combines with NF-AT_n to help drive expression of the IL-2 gene (for review, see 99, 107).

This model is certainly an oversimplification of the finely coordinated and complex sequence of parallel pathways engaged during T cell activation; it may, however, provide a useful framework for further studies of the roles of ion channels in shaping these events. As we attempt to describe the ionic signaling events that couple antigen recognition to the control of gene expression, important gaps in our understanding become apparent. The specific functions of K^+ channels in lymphocytes are not yet fully understood, nor have the ramifications of K^+-channel diversity, modulation, and developmental regulation been explained. Basic features of depletion-activated Ca^{2+} channels are obscure; how are these channels controlled at a molecular level, and how does feedback regulation of their activity create $[Ca^{2+}]_i$ oscillations? Finally, the meaning of $[Ca^{2+}]_i$ oscillations is a mystery. Do oscillations encode specificity by selecting certain response pathways over others, or are they simply a means of enhancing the efficiency of signal transduction under conditions of weak antigenic stimulation? Application of increasingly sensitive techniques of molecular physiology will help us answer these questions and unravel the intricate web that connects ion channels, intracellular messengers, and gene transcription in lymphocytes.

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